Composite Tissue Allotransplantation by Modulation of Immunosuppression

Larsen, M.

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Composite Tissue Allotransplantation by Modulation of Immunosuppression

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VRIJE UNIVERSITEIT

Composite Tissue Allotransplantation by Modulation of Immunosuppression

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad Doctor aan
de Vrije Universiteit Amsterdam,
op gezag van de rector magnificus
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ten overstaan van de promotiecommissie
van de faculteit der Geneeskunde
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door

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geboren te Nairobi, Kenia

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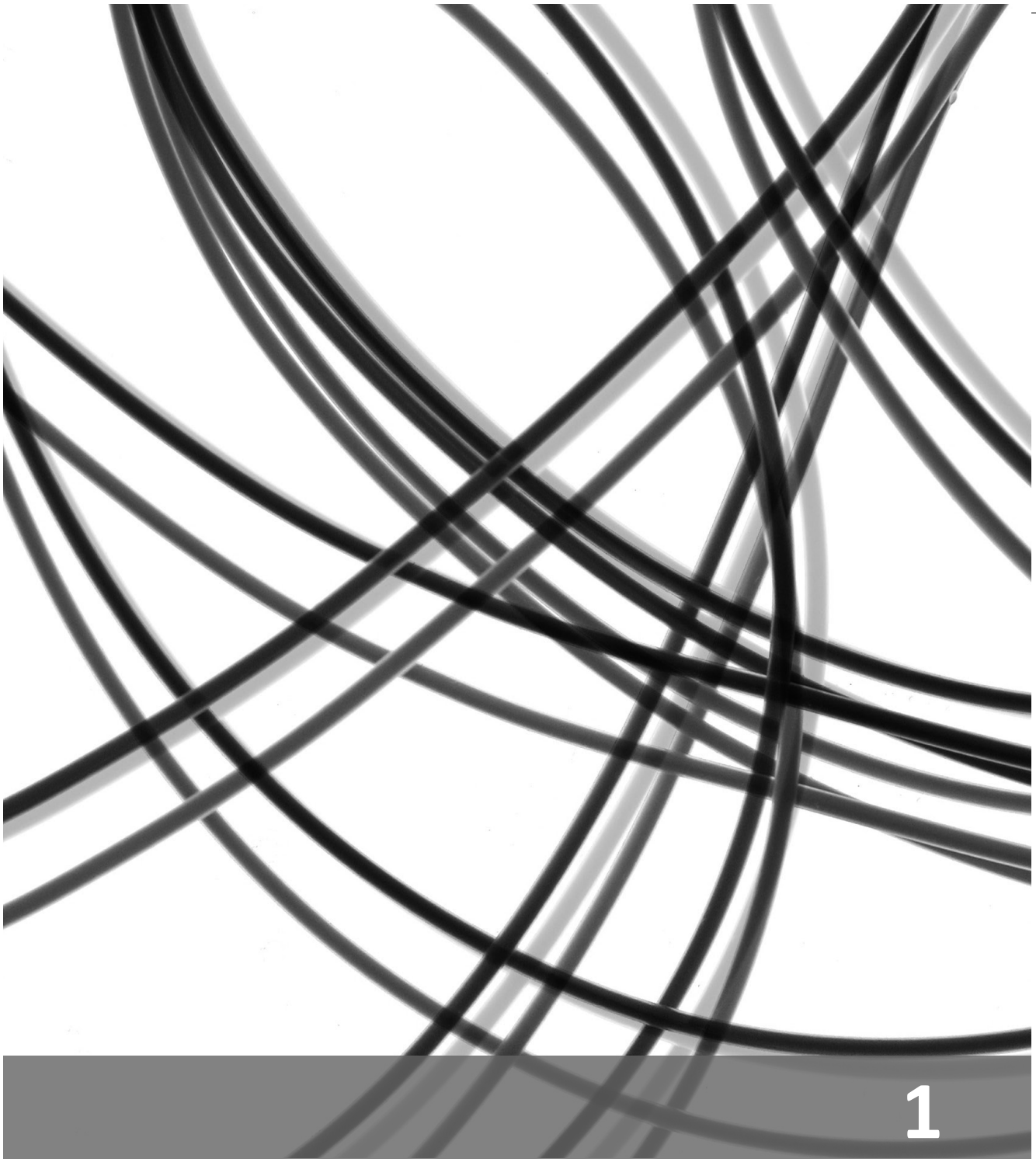
copromotor: dr. H.A.H. Winters

To my dear wife Katarína, and our son William

To my parents

To the animals

Contents



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INTRODUCTION

A. Specific Aims

Segmental loss of bone or joint is a difficult problem for which current reconstructive methods often fail. Structural allografts are only incompletely revascularized, and are prone to nonunion, late stress fracture and unpredictable healing. Vascularized autografts provide superior ability to heal and remodel with applied stress, but are frequently of insufficient size, shape and strength. Prosthetic replacement of diaphyseal bone may fail, loosen or produce periprosthetic fractures[1-4]. Engineered bone replacements remain largely experimental.

Transplantation of *living* allogenic bone would provide a replacement closely matched to the dimensions and mechanical properties of the resected bone, combined with the desirable healing properties of a vascularized autograft. Maintaining tissue viability in the face of acute and chronic rejection remains a challenge however, requiring long-term immunosuppression or induction of tolerance with attendant significant risks. Methods permitting the use of vascularized (living) bone or composite tissue allotransplants (CTAs) without the health risks of immunosuppressive drugs or tolerance induction would be an important advance.

The overall goal of this research is to maintain viability of microsurgically transferred vascularized bone allotransplants without the need for long-term immunosuppression or tolerance induction. We propose a novel method by which this may be accomplished: combined microsurgical bone transfer and the development of a new osseous blood supply of host origin. Surgically implanted host arteriovenous bundles will promote neoangiogenesis. As these new host-derived vessels are non-immunogenic, only short-term immunosuppression is necessary, allowing sufficient time for angiogenesis to occur.

Hypothesis 1: Neoangiogenesis from implanted host vessels will maintain blood flow to a vascularized bone allotransplant after withdrawal of immunosuppression (Chapters 2 and 3).

Specific Aim 1: To test whether blood flow is preserved in vascularized bone allotransplants after surgical implantation of host arteriovenous (AV) bundles, and following withdrawal of short-term FK-506 immunosuppression.

We will transplant vascularized bone allotransplants across a major histocompatibility barrier using sex-mismatched Dark Agouti (DA) female rat donors and Piebald Virol Glaxo (PVG) male recipient rats, implanting a saphenous AV bundle within the medullary

canal to provide host-derived angiogenesis. Short-term immunosuppression will be used. We will ask if nutrient vessel and implanted host vascular bundle patency is maintained, and correlate these findings with measured bone blood flow (hydrogen washout technique) and extent of neoangiogenesis (microangiography). We will correlate the extent of neoangiogenesis with immunosuppression and immune rejection.

Hypothesis 2: Vascularized bone allotransplants will retain the ability to synthesize and remodel bone matrix after neoangiogenesis and subsequent withdrawal of immunosuppression (Chapter 4).

Specific Aim 2: To test whether bone remodeling is maintained in vascularized bone allotransplants after surgical implantation of host AV bundles and withdrawal of short-term FK-506 immunosuppression.

We will quantify the amount of new bone formation and resorption by histomorphometric analysis.

Hypothesis 3: Donor specific tolerance does not contribute to graft survival after withdrawal of immunosuppression (Chapter 4).

Specific Aim 3: To test whether vascularized allotransplant survival is the result of donor-specific tolerance, following implantation of host AV bundles and withdrawal of short-term FK-506 immunosuppression.

We will compare allotransplant survival to non-viable allografts and isotransplants. We will determine patency and blood flow. We will test whether tolerance has occured by determining the fate of a genetically identical skin graft placed at a second stage. These data combined will serve as a validation of the method.

Hypothesis 4: The rate of angiogenesis and subsequent bone remodeling can be favorably modulated with growth factor administration (Chapters 5 and 6).

Specific Aim 4: To test whether local delivery of basic fibroblast growth factor (FGF2) and/or vascular endothelial growth factor (VEGF) results in improved measures of capillary formation, bone remodeling and bone blood flow.

At surgery, we will administer biodegradable microspheres encapsulating FGF2, VEGF, or both within the transplanted femur adjacent to the AV bundle. We will measure the effect of the growth factor(s) on angiogenesis, bone remodeling and bone blood flow.

Hypothesis 5: Growth factor administration and AV bundle implantation can augment bone blood flow and bone formation in conventional allografts (Chapter 7).

Specific Aim 5: To test whether local delivery of FGF2 and/or VEGF results in similarly improved measures of bone viability in conventional bone allografts.

We will administer microspheres encapsulating FGF2 and/or VEGF adjacent to the AV bundle implanted in a previously frozen femur allograft. Angiogenesis, bone remodeling and bone blood flow will be measured. If successful, this model is readily applicable to the clinical situation and will serve as a means to assess the clinical use of an AV bundle with or without microspheres inside bone allografts before application to vascularized allotransplants.

Hypothesis 6: New bone formation in transplanted allogeneic bone is the result of graft chimerism (Chapter 8).

Specific Aim 6: To evaluate whether osteocytes in newly formed bone are of recipient rather than transplant origin.

Transplanted (female DA) bone specimens removed from male PVG recipient animals will be studied at two time points. Areas of new bone will be identified by fluorochrome labeling. Osteocytes from this area will be sampled by laser capture microdissection and analyzed with a quantitative real-time polymerase chain reaction (qRT-PCR) for the SRY gene (Y-chromosome specific) and the cyclophilin housekeeping gene to test our hypothesis.

Hypothesis 7: Surgical angiogenesis will preserve viability, permit healing and maintain articular function of whole joint allotransplants (Chapter 9).

Specific Aim 7: To evaluate the ability of host-derived neoangiogenesis to preserve viability, healing potential and function of whole knee joint composite tissue allotransplants (CTA) when placed orthotopically in the hind limb of PVG rats.

We will orthotopically transplant a whole rat knee CTA with microsurgical anastomoses. Simultaneously, an epigastric fascia flap will be placed in the femur and a saphenous AV bundle in the tibia. Short-term immunosuppression will maintain nutrient pedicle flow during angiogenesis. We will evaluate the viability of femoral and tibial bone, hyaline cartilage and joint capsule by histology and correlate these finding with mechanical properties of joint movement, weight bearing, and bone blood flow as measured by hydrogen washout.

Hypothesis 8: Routine measurement of small bone blood flow is feasible and safe with the hydrogen washout method (Chapter 10).

Specific Aim 8: To evaluate the feasibility and safety of routine use of the hydrogen washout method to measure cortical bone blood flow in the laboratory rat.

Hydrogen washout is a method used to measure rates of change in hydrogen concentration in a tissue or fluid. We will modify its use to the laboratory rat by developing software and measurement apparatus, combined with commercially available sensors and a hydrogen generator, to allow safe and repeated ease of use.

Hypothesis 9: The hydrogen washout method of bone blood flow measurement is sensitive to flow changes, and agrees well with the gold standard, radioactive microsphere entrapment (Chapter 11).

Specific Aim 9: To compare bone blood flow measurements obtained by hydrogen washout to the radioactive microsphere entrapment method.

Bone blood flow measurements in New Zealand White rabbits will be compared when obtained by hydrogen washout and by radioactive microsphere entrapment. Blood flow will be altered by systemic administration of vasoactive drugs. Repeated measurements on pedicled rat femora will be obtained to assess the correlation between first and subsequent bone blood flow measurements. These steps allow the validation of the hydrogen washout method for its use in bone tissue, which has not yet been performed.

B. Background and Significance

B.1.The Clinical Problem: Segmental bone and joint loss

The segmental loss of bone and/or joint may result from resection of primary and metastatic tumors, congenital deficiency, trauma, infection or failed prosthetic implant arthroplasty. Currently available reconstructive options include cryopreserved allotransplants, vascularized bone autografts, bone transport and prosthetic replacement. All have significant problems, discussed below. Future reconstruction of the axial skeleton may well make use of additional methods, such as tissue engineering or direct replacement of ‘like with like’, using vascularized (living) bone or composite allogeneic tissue.

B.1.a. Cryopresered structural allograft reconstruction

Cryopreserved structural bone allografts are widely used for reconstruction of segmental loss. Although they contain no viable cells, a host immune response occurs which includes development of anti-HLA antibodies[5,6]. Immunogenicity is diminished by freezing among other methods, or eliminated by immunosuppresive drugs[7-14]. They provide immediate stability when combined with internal fixation and closely match defect bone size and shape. Healing of nonviable bone occurs by creeping substitution. The process is slow and incomplete, mostly seen at the allograft/host junctions and periosteal surfaces. Forty to 50% of lamellar bone remains necrotic, and bone resorption results in significant mechanical weakening at 6 to 12 months[15]. Repair and remodeling responses remain essentially non-existent[16]. Thus, cryopreserved allografts have a significant incidence of stress fracture and nonunion[17] (27% and 49%, respectively in a recent large series[18]). Fracture requires revision, and infection is another risk (13.3% in a recent study[19]). It is catastrophic, requiring allograft removal with risk of recurrence at re-implantation[5,6,20].

Summary:

> Cryopreserved allograft bone provides stability and good size/shape match of skeletal defects. Healing is slow, bone replacement incomplete, with little or no repair and remodeling capability. Fracture or infection requires implant removal.

B.1.b. Vascularized bone autograft reconstruction

Vascularized, or living bone transfers have been used since 1905[21-23]. Multiple sources have been used, including rib, fibula, iliac crest, scapula, radius, and femoral condylar or trochanteric bone[24]. Osteocytes and other cellular components remain viable, thus obviating creeping substitution. No osteopenia occurs, and strength, and healing are thus superior to other methods[25,26]. Hypertrophy in response to stress also occurs[27-30]. Vascularized bone transfer is indicated for defects greater than 6 to 8 centimeters, or when healing potential is diminished by radiation, scarring, infection or osteonecrosis[25,28,31,32]. Restoration of longitudinal growth by inclusion of growth plates[33-37], revascularization of necrotic bone[25,32,38-47], improvement of local blood flow in scarred beds[48,49] and/or reconstruction of composite tissue loss is possible in one procedure. Limited donor site availability, attendant donor site morbidity,

and size/shape mismatch at the recipient site are frequent problems, however. Expendable sources are limited, primarily to fibula, rib and iliac crest for large defects[28,50-56]. They are often poor structural replacements for metaphyseal defects, and are grossly mismatched in size for humerus, femur and tibia diaphyses[50-52]. Load sharing by rigid internal fixation, external fixators, and/or structural allografts is recommended, along with postoperative protection[28,50]. A primary union rate of 61% has been reported[28]. All vascularized transfers currently are autogenous, due to the impracticality of maintaining long-term immunosuppression for non-life-critical tissue allotransplantation.

Summary:

Living bone heals rapidly and may remodel and heal in response to stress. Donor sites are limited, and size and shape mismatch remains a problem for most areas of segmental loss.

B.1.c. Other methods of reconstructing segmental loss

Prosthetic reconstruction of diaphyseal or periarticular defects allows immediate functional restoration[1,57-60], but has significant complication rates due to mechanical failure[61]. Reimplantation of resected tumor bone has been reported[62]. The method requires devitalization of cells prior to replacement by autoclaving[62-67], pasteurization[68,69], irradiation[70], or cryotherapy[71]. While local recurrence has been low[62], stress fracture, infection, bone resorption and prosthetic/bone loosening occurs frequently[63]. Tissue engineering, the creation of bone from matrix, cells and growth factors, has been demonstrated in the laboratory by use of vascular bundles or periosteum place into or around bone fragments, demineralized bone matrix, hydroxyapatite or polyethylene chambers[72-75]. Muscle may also be transformed using silicone rubber molds containing growth factors[76]. The method remains primarily experimental[77,78], and does not permit reconstruction of more complex tissues including articular surfaces, ligament or tendon.

Summary:

Prosthetic reconstruction of segmental defects have high rates of failure. Reimplanting devitalized tumor bone has risks similar to nonviable allograft, and a small risk of reimplanting viable tumor cells. Tissue engineering of bone remains a laboratory exercise at present.

B.2. Living bone allotransplants

Transfer of living allogenic tissues would combine the desirable biology of living bone with close matching of defect size, shape and tissue type, and would eliminate donor site morbidity. Mechanisms for identifying and matching potential donors to recipients exist currently in multi-organ donation programs. As bone is ischemia tolerant, tissue harvest may take place after cardiac activity has ceased, and tissue viability maintained for up to 5 days in cold storage[79-84]. While microvascular surgery makes such procedures technically feasible, issues related to immunogenicity make them experimental at present. Vascularized bone and joint allotransplants have been performed clinically, however, including femoral segments[85], fibula[86], and a series of six whole knee joint transfers[87-90].

Summary:

Living allotransplanted tissue combines the benefits of nonviable allograft stability, size and shape with the biologic properties of living bone. Only a few such cases have been reported.

B.3. Immune modulation in composite tissue allotransplantation

Clinical use requires a method that maintains tissue viability long-term, including measurable blood flow, osteocyte viability, active bone remodeling and healing response, and maintained biomechanical properties. Safety in such non-life-critical tissues is paramount. Current immune modulation methods carry significant risks, including graft-versus-host (GVH) disease, opportunistic infection and carcinogenesis. Most published research makes use of immunosuppressive drugs and/or efforts to induce a tolerant state. The possibility of eliminating such long-term immune modulation by development of a neoangiogenic host circulation within the transplanted bone is the focus of this research.

Summary:

Risks of immunosuppression or tolerance induction are significant and difficult to justify in non-life-critical tissue transplantation such as bone, joint or other musculoskeletal composite tissue.

B.3.a. Drug therapy

Experimental studies demonstrate musculoskeletal tissues to be more challenging than solid organ transplants, requiring 2-3 times greater immunosuppressive doses. Of the component tissues, skin, muscle, and vascular endothelium are most immunogenic[91]. Rejection causes damage to vascular endothelium, with resulting increased vascular permeability and activation of leukocytes[92]. Vessel thrombosis and tissue death follows. Cylosporin A (CsA) inhibits the release and utilization of interleutin-2[93], and has maintained vascularized bone allograft viability in canines[94]. Benhaim has found a combination of CsA and mycophenolate mofetil to be superior to CsA alone in a rat hind limb model[91]. Mycophenolate mofetil reduces the availability of guanosine nucleotides, and thus inhibits cellular division, particularly in T and B lymphocytes[95]. Drug therapy does not reliably control chronic rejection, however. This remains the major cause of allograft loss[96]. Additionally, long-term drug use carries risks including delayed wound healing, skin malignancies, low-grade lymphomas, lymphoproliferative disorders, and end-organ toxicity[97-99].

Summary:

> Musculoskeletal tissues are highly immunogenic. Difficulty in controlling chronic rejection, and complications related to long-term drug use carry significant risks.

B.3.b. Tolerance induction

Donor-specific tolerance is a state of unresponsiveness to specific donor antigens. The concept of engraftment of donor bone marrow cells in host tissue (mixed chimerism) to induce donor-specific allograft tolerance was first proposed in the 1950s[100,101]. Such survival of donor lymphoid cells has required fully ablative or lethal conditioning. Thymic clonal deletion of donor responding immunocytes[102], use of costimulatory blockade[103,104], low-dose radiation or antilymphocyte serum (ALS), combined with drug therapy[105-109] or infusion of donor bone marrow (BM)-derived cells combined with ALS have shown some ability to induce tolerance in skin and composite tissue transplants for a period of time[97,110]. A more gentle conditioning, using intraosseous transplantation of purified $CD90^+$ HSC cells at the same time as limb transplantation has demonstrated successful short-term allogeneic hind-limb survival without supplemental immunosuppression[111]. Recipient conditioning

to encourage long-term or even indefinite survival of donor stem cells should be minimally toxic to be practical. Complications of recipient conditioning protocols are the most serious problems in induction of tolerance, and include graft-versus-host (GVH) disease. GVH is a potentially lethal attack of the immune system by engrafted chimeric donor cells[112-114]. The complications of tolerance induction are as onerous as those of immunosuppression. With current techniques, neither drug therapy nor tolerance induction are practical for clinical use. Other possibilities must therefore be considered.

Summary:

An alternative to drug therapy is induction of tolerance. Mixed chimerism, required for this process, has required lethal conditioning, and successful engraftment risks graft-versus-host disease. Less stringent methods are under investigation.

B.3.c. Graft adaptation

Another mechanism allowing allotransplant survival is *graft adaptation*[115]. Defined as allotransplant survival in a non-tolerant and immunologically competent host[116], it is possible if vascular endothelium is replaced by host derived cells[117-119], decreased blood flow[120], loss of passenger leukocytes, decreased expression or masking of the incompatible antigens, or presence of suppressor cells or factors in the graft[121].

B.4. Host-derived neoangiogenesis

We describe a novel method to enable transplantation of living bone without long-term immune modulation. The method replaces endosteal circulation with a host-derived neoangiogenic blood supply. This is accomplished by placing vascularized recipient tissue within the bone at the time of transplantation, together with microvascular repair of nutrient vessels. Only short-term immune modulation is necessary while neoangiogenesis from host tissue occurs. Thereafter, rejection and resulting nutrient vessel thrombosis may have little effect on bone blood flow.

Angiogenesis is a biologic process of new capillary formation[122-124]. Its regulation is complex, mediated by growth factors, interactions with adjacent cells or matrix, and proteolytic enzymes that modify regulatory molecules, cell surface molecules, and the extracellular matrix. *Therapeutic angiogenesis* describes the induction or stimulation

of neovascularization for treatment of pathological clinical situations characterized by local hypovascularity[125,126]. Surgical transfer of vessels or well-vascularized autogenous tissue, referred to as *surgical angiogenesis*[127,128] is an example, used alone or augmented by vasculogenic growth factors. Surgical angiogenesis in autogenous bone was first demonstrated by implantation of an arteriovenous (AV) vascular bundle in a canine tibia[128]. Subsequently AV bundles have been shown to induce new bone formation in autograft[25,127,129-133], allograft[131,132], and xenograft bone[134]. Surgical angiogenesis has been applied clinically in the treatment of Kienbock's disease[135,136], talar avascular necrosis[128], scaphoid nonunion with avascular necrosis[31,137-142], and in prefabricated bone free flaps[76,143,144]. Its potential application to maintain vascularized allotransplant is our research focus.

Summary:

New capillaries sprout from vessels implanted into ischemic tissues by angiogenesis. When placed into necrotic bone, vascularized tissue results in new bone formation. We propose to use this method to maintain allotransplanted bone and joint viability.

B.4.a Growth factors in tissue transplantation

B.4.a.1 Angiogenic growth factors

Basic fibroblast growth factor (FGF2) is a powerful stimulator of neoangiogenesis *in vivo* and a pleiotropic regulator of vascular cell proliferation and migration *in vitro*[145]. It promotes formation of larger and more complex blood vessels such as arterioles as well, stimulating migration and proliferation of smooth muscle, endothelial and fibroblast cell types, and in bone simultaneously promotes osteoid formation[146]. Other angiogenic growth factors, such as VEGF, are specific only for endothelial cells. We have reported VEGF to promote angiogenesis in AV bundles implanted into necrotic bone, when delivered directly or with endothelial cell transfection[131,133]. FGF2 has also been used successfully in necrotic autogenous bone[145] and in prefabricated hydroxyapetite molds[146]. VEGF works synergistically with FGF2 to stimulate angiogenesis *in vitro*[147] and *in vivo*[148]. Vascular remodeling in response to these growth factors is further dependant on arterial sufficiency and nitric oxide production[149]. Their use to improve the neoangiogenesis of implanted AV bundles or facial flaps in our model of bone and CTA transplantation merits investigation.

Summary:

VEGF and FGF2 promote neoangiogenesis *in vivo* and have been shown to improve blood flow in bone when combined with surgical implantation of AV bundles.

B.4.a.2 Bone-forming growth factors

Bone morphogenic proteins (BMPs) are low molecular weight glycoproteins belonging to the TGF-β family[150]. Of all the growth factors present in bone, they are the only ones known to induce maturation of undifferentiated mesenchymal cells into osteocytes, (*osteoinduction)*[151]. BMP-2 has been used extensively in experimental and preclinical studies of bone regeneration[152-156], demonstrating ability to heal experimental skeletal defects as effectively as autografts or allografts. A prerequisite for BMP function is availability of target cells. That is, BMP is not effective in a non-vascularized environment. Further, an osteoconductive matrix, serving as a scaffold for cell growth is required[155]. While recombinant human (rh) TGF- β 1 and FGF2 also have some ability to form bone, rhBMP-2 remains the most effective promoter[157]. In a study of autoclaved resected bone reimplantation, FGF2 improved the remodeling process when compared to autoclaved bone alone[158]. A synergistic effect on bone formation has been shown by simultaneous use of TGF- β3[159], but not TGF- β1[160]. It is reasonable to surmise a possible role for growth factors in improving bone remodeling and bone material properties after bone or CTA transplantation.

Summary:

BMPs are glycoproteins that transform undifferentiated mesenchymal cells into osteocytes. They have been shown to heal bone defects effectively, but require living target cells and an osteoconductive matrix to function.

B.4.b. Local growth factor delivery

Growth factors may be administered by injection in a single bolus, with osmotic pumps[133], or with use of gene therapy via replication-deficient adenovirus, liposomes or gene-coated balloon catheters[131-133]. Local administration of vascular growth factors significantly promotes angiogenesis. A steady-state release is more effective in doing so than a bolus injection[161,162]. One method by which continuous administration may be achieved is via poly(D,L-lactide-co-glycolide) (PLGA) biodegradable

microspheres[163]. Using a solid-encapsulation/single-emulsion/solvent extraction method, growth factors in solution may be encapsulated within a biodegradable polymer[163]. The method significantly lowers dosages necessary to achieve physiologic effect and protects and maintains the growth factor *in situ,* allowing interaction with target cells. The manufacturing technique is straightforward[164]. It permits growth factors such as FGF2, whose half-life is 50 minutes *in vivo*, to affect tissues that generally require 24 hours of exposure for response[165]. PLGA microspheres have been characterized by our co-investigators to gradually degrade over a 28-day course[166]. VEGF released from microspheres was detectable by enzyme-linked assay and was shown to maintain *in vitro* bioactivity in cell culture proliferation assays[163]. The *in vitro* concentration of VEGF required for bioactivity is approximately 10 ng/mL[167]. Similar values are estimated for FGF2, with higher levels inhibiting osteogenesis[168]. By optimizing polymer viscosity, growth factor loading volume and vortexing speed, loading efficiencies of approximately 80% and zero-order drug release of vascular growth factors and BMP are achievable[166,169].

Summary:

Therapeutic use of growth factors requires local delivery. Single bolus injection, osmotic pumps, local gene therapy and use of PLGA biodegradable microspheres are possible methods.

B.5. Clinical significance

Segmental bone defects are commonly encountered in orthopedic practice, resulting from traumatic loss, osteomyelitis, failed arthroplasty and tumor surgery. Cryopreserved allograft bone is frequently used for the reconstruction, and is matched to fit the defect. Late stress fracture, infection and nonunions are frequent complications. Living autogenous bone, whose circulation in maintained by microvascular repair, can adapt to stress and heals more readily. Poor matching of dimension and strength limits their potential. A living allotransplant, combining the best characteristics of both seems ideal, but unacceptable problems associated with immunosuppressive drugs or tolerance induction makes their use impossible. Surgical angiogenesis combined with short-term immunosuppression may maintain viability of a vascularized bone allograft without need for immunosuppression or induction

of tolerance. This is the key focus of our research. We will investigate methods to enhance angiogenesis and bone remodeling by growth factor administration, further refine our understanding of osteocyte lineage (graft chimerism) in newly formed bone, and demonstrate the feasibility of the method to transplant composite whole joint allotransplants. We will adapt and validate methods of bone flood flow measurement to routine laboratory use. An evaluation of the risks of short-term immunosuppression, as explained by a clinical case (Chapter 12), is also provided.

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2

HOST-DERIVED ANGIOGENESIS MAINTAINS BONE BLOOD FLOW AFTER WITHDRAWAL OF IMMUNOSUPPRESSION: DEVELOPMENT OF A NOVEL METHOD OF COMPOSITE TISSUE ALLOTRANSPLANTATION

Introduction

Large skeletal defects resulting from resection of bone tumors, infection or failed arthroplasty present a reconstructive challenge. Available reconstructive methods have limitations. Structural *allografts* (defined as allogeneic bone without blood supply or viable osteocytes) are incompletely revascularized[1], prone to nonunion[2], and late stress fracture[3]. Prosthetic replacement of diaphyseal bone may fail, loosen or produce periprosthetic fractures[4-7]. Engineered bone replacements remain largely experimental.

Another alternative is the use of bone transplantation, in which the tissue is placed in a new recipient site with its circulation and osteocyte viability intact. Autogenous bone transplantation, commonly from the fibula as a free bone flap is a common example. Bone may also be transplanted from a different donor of the same species. This is not a grafting procedure, but rather a form of composite tissue allotransplantation (CTA) with tissue limited to bone and its nutrient vessels. In this thesis, for clarity we will refer to such living tissue transfers as either *allotransplants* or *isotransplants*, depending upon their MHC match with the recipient animal. For clarity, the term *allograft* will specifically refer to nonviable, previously frozen bone implanted without any native vascular supply.

Vascularized bone transplants are superior to bone grafts in both ability to heal and remodel with applied stress, but expendable autogenous sources are frequently of insufficient size, shape and strength. Expendable sources are limited, with fibula and iliac crest used for larger defects. These bone flaps are generally poor structural replacements for segmental loss of the humerus, femur and tibia. Humeral shaft and distal humerus reconstruction with autogenous fibula transplantation, for example, has a high rate of complications[8].

Transplantation of living allogeneic bone could provide a replacement closely matched to the dimensions and mechanical properties of the resected bone, combined with the desirable healing properties of autotransplants. Few such procedures have been performed clinically as they require long-term immunosuppression to maintain circulation and viability[9,10]. The results in human trials have been disappointing. A long-term review of femur and knee allotransplants found five of six cases to have failed[11]. One transplant has maintained viability using long-term Tacrolimus (FK-506, a macrolide immunosuppressant drug) and mycophenolate mofetil

immunosuppression. Methods permitting vascularized bone allotransplantation without the health risks of immunosuppressive drugs or tolerance induction would be an important advance.

The presented research investigates a novel alternative to long-term immune modulation, using principles of therapeutic angiogenesis to create a new autogenous circulation within the allogeneic bone transplant. At surgery, after repair of the allogeneic nutrient vessels, vascularized tissue consisting of an arteriovenous (AV) bundle is inserted within the bone. Short-term immunosuppression is used to maintain nutrient blood flow until the new autogenous circulation develops from the implanted vessels. Following withdrawal of drug therapy, long-term viability of the transplanted bone is maintained despite nutrient vessel thrombosis.

Angiogenesis is the growth and proliferation of blood vessels from existing vascular structures, and is fundamental to reproduction, development, and repair of tissue. In bone, such vessels play an important role in osteogenesis[12]. The term *therapeutic angiogenesis* describes the induction or stimulation of neovascularization for the treatment or prevention of pathological clinical situations characterized by local hypovascularity. Neovascularization in necrotic bone has been induced in both experimental and clinical studies by direct blood vessel implantation[13-18].

In organ transplantation, withdrawal of an immunosuppressant leads to vascular thrombosis and resultant loss of tissue viability and function. In this initial short-term study, we demonstrate that neoangiogenesis from a host-derived AV bundle maintains measurable bone blood flow and tissue viability despite vascular pedicle thrombosis following withdrawal of immunosuppression.

Materials and Methods

Animals and experimental design

Vascularized allogenic femoral bone transplantation was performed from female DA rats (168 ± 31g body weight: mean ± standard deviation) with the major histocompatibility antigen (MHC) RT1^a (as the donor) to age-matched PVG male rat recipients (213 ± 29 g body weight) with MHC RT1^c (as the recipient). The haplotypes of the rat groups used were DA (RT1.AaBa) and PVG (RT1.AcBc), comprising a full MHC mismatch. All animals were purchased from the same supplier (Harlan

Sprague Dawley, Madison, WI). Vascularized bone allogeneic transplantation was performed in 97 rats. Rats were randomly allocated to one of 6 groups: (I) No immunosuppression, patent AV bundle (n = 23), (II) No immunosuppression, ligated AV bundle (n = 11), (III) FK-506 immunosuppression for 2 weeks, patent AV bundle (n = 19), (IV) FK-506 immunosuppression for 2 weeks, ligated AV bundle (n = 14), (V) FK-506 immunosuppression for 2 weeks, with additional 2 week survival, patent AV bundle (n = 19) or (VI) FK-506 immunosuppression for 2 weeks, with additional 2 week survival, ligated AV bundle (n = 11). The survival period was 2 weeks for groups I, II, III, and IV and four weeks in groups V and VI. When used, FK506 (Fujisawa Pharmaceutical Co., Osaka, Japan) was injected intramuscularly once daily at a dose of 1 mg/kg. This experiment was reviewed and approved by the Institutional Animal Care and Use Committee, and carried out following institutional animal care guidelines.

Sterile technique was maintained throughout the procedures. Donor animals were anesthetized with pentobarbital sodium (35 mg/kg IP) for the non-survival harvest surgery. Euthanization was by intracardiac injection of Beuthanasia (Sleepaway; Fort Dodge Animal Health, Fort Dodge, IA, 200 mg/kg). Recipient animals were anesthetized with a combination of ketamine (90 mg/kg IM), and xylazine (10 mg/kg IM). If necessary, during the surgical procedure the animal was re-dosed with 20 mg/kg IP injections of ketamine. Fragmin (10 IU/day) and Butorphanol (0.05 mg/kg/day) were given for five days post-operatively. Staples and sutures were removed 7-10 days post-operatively. Animals were allowed to move freely in their cages and fed standard rodent feed and water *ad libitum*.

Animal Model

Microvascular transplantation of the rat femur was first described in 1984 based on prior anatomical dissections[19]. The vascular distribution to the bone is provided by proximal and distal nutrient branches arising from the lateral femoral circumflex artery and femoral artery, respectively (Figure 1).

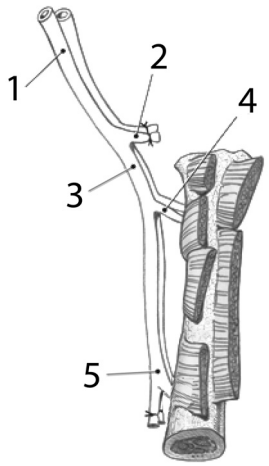

Figure 1. Drawing of the rat femoral graft with (1) common iliac a. & v., (2) ligated superficial circumflex iliac a. & v., (3) lateral femoral circumflex a. & v. with proximal nutrient branch to femur (4), femoral a. & v. with distal nutrient branch (5).

Operative Procedure

Donor femur harvest

Under anesthesia, the right femoral and lateral femoral circumflex vessels were exposed. We used both the proximal and distal nutrient vessels to the femur, which branch from these vessels and pass between the pectineal muscle and the medial vastus muscle into the femoral shaft. To facilitate microvascular anastomoses, the vessel dissection was extended proximally to include the common iliac artery and vein. The femur was then further dissected from surrounding tissues and disarticulated at the hip and knee joints. The femoral head and neck and distal femoral condyles were resected and the medullary canal reamed by hand with a 2 mm drill. The femoral transplant, once removed, was irrigated with heparinized saline and cooled during the preparation of the recipient site. The transplant measured approximately 20 mm in length.

Transplantation procedure

The femur was transplanted heterotopically to a subcutaneous abdominal pocket, revascularized by repair of the nutrient circulation. Figure 2 is a schematic representation of the procedure. A saphenous AV bundle was raised from the left hind limb of the recipient animal and placed within the medullary canal. The implanted AV bundle was in fact a small pedicled fascial flap raised from the left leg including the saphenous artery and venae comitantes (Figure 3). Circulation was restored to the transplanted femur by end-to-end repair of its pedicle (common iliac artery and vein) to the femoral vessels just proximal to the origin of the saphenous vessels using 11-0 and 10-0 nylon sutures for the artery and vein, respectively. No vascular compromise to the hind limb was seen postoperatively as sufficient nutrition is provided through pudendal branches and those traveling with the ischial nerve.

The abdominal location permitted isolation of the transplant from surrounding tissue by envelopment in a 0.18 mm thick reinforced silicone sleeve (Bentec Medical, Woodland, CA), enabling a more accurate assessment of blood flow differences between groups (Figure 4). The AV bundle was pulled through the medullary canal to fill its entire length and fixed with an 8-0 nylon suture. The AV bundle was ligated at this point in Groups II, IV and VI. Immunosuppression dosing parameters were based upon our previous research, which found two weeks' administration to allow sufficient AV bundle angiogenesis to maintain blood flow and osteocyte viability after subsequent nutrient pedicle thrombosis[20].

Preparation for Transplant Harvest

We used two methods to evaluate the circulatory status of the bone. Local cortical bone blood flow was measured quantitatively by the hydrogen washout method, and the extent of neoangiogenesis within the bone from the implanted autogenous AV bundle was quantified by measuring capillary density. Viability of osteocytes was assessed by a count of empty versus occupied lacunae.

In a nonsurvival operation, anesthesia was induced. Arterial blood pressure was continuously monitored via a carotid artery catheter connected to a transducer (Transducer Pressure Monitoring Kit, Edwards Lifesciences, Irvine, CA; LabVIEW™ software, National Instruments, Austin, TX). A 14-gauge catheter connected to a T-tube was inserted through a tracheotomy. One end of the T-tube was connected via

a Y-connector to an oxygen source and a hydrogen generator (Whatman Hydrogen Generator 75-32, Whatman Inc. Haverhill, MA).

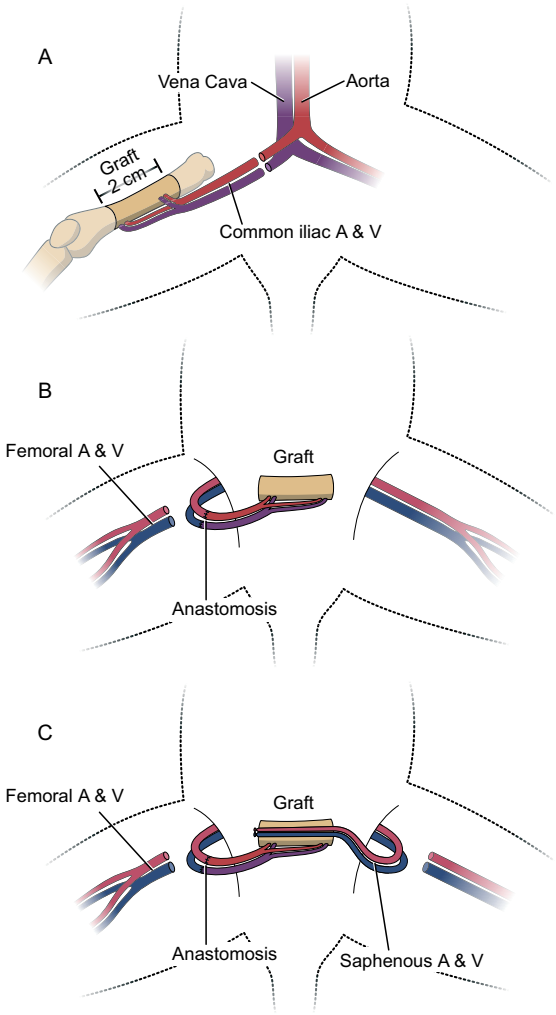

Figure 2. Diagram showing the surgical technique. (A) Donor procedure; resection of the femur diaphysis based on the nutrient vessels to the bone. (B) Microvascular anastomosis in the recipient; the slight size mismatch in donor and recipient animals permitted matching of the donor common iliac vessels to the recipient femoral vessels. (C) AV bundle implantation; with the transplanted bone in the abdominal pocket, the recipient saphenous vessels were carefully pulled through the medullary canal and fixed to the abdominal wall.

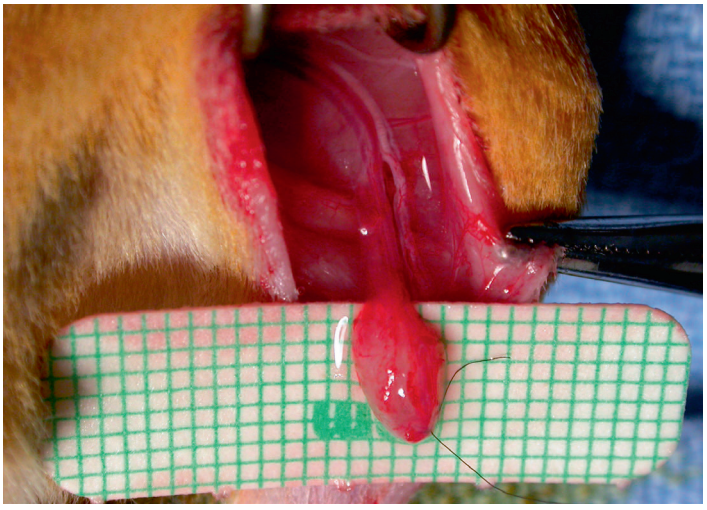

Figure 3. Picture of the AV bundle dissection in the left leg in the recipient. A 3 x 5 mm distal fascial flap is elevated on the saphenous artery and venae commitantes to the bifurcation in the groin. This permitted enough vascularity and length to assure proper placement and survival inside the bone.

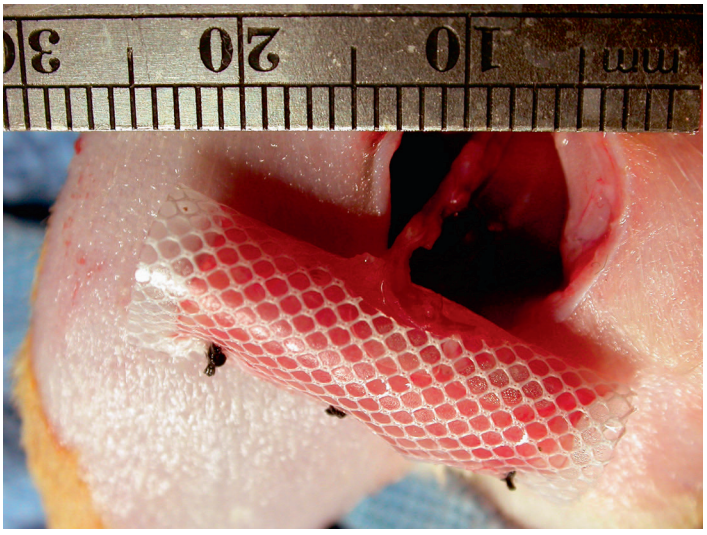

Figure 4. Picture of the graft after vessel anastomosis in the recipient's right groin, and wrapping in a reinforced silicone membrane. After this, the graft was placed in a subcutaneous abdominal pocket and the AV bundle pulled into the medullary canal.

Bone blood flow measurement

The femoral transplant or graft was exposed through a transverse lower abdominal incision and by careful trimming of the silicone membrane. The patency of the microvascular anastomosis and AV bundle were evaluated. A patent vessel was observed to have both visible gross pulsation and a positive standard microsurgical patency test. Cortical blood flow was determined using the modified hydrogen washout method we described and validated previously (see Chapters 10 and 11, respectively)[21,22]. Briefly, a shallow hole was drilled perpendicularly into the cortical bone. A hydrogen sensor (H2-50, Unisense, Aarhus, Denmark) connected through a picoammeter (Picoammeter2000, Unisense, Aarhus, Denmark) to a data acquisition interface (DAQpad-6020E, National Instruments, Austin, TX) was placed in the hole with a micromanipulator. Hydrogen was added to the breathing mixture (30% oxygen and 70% hydrogen) and tissue hydrogen saturation achieved as determined by a steady-state sensor output. Blood flow was then calculated by the rate of hydrogen washout from bone after the hydrogen generator was turned off using the method and equation of Whiteside et al.[23,24]: $C = C_0 e^{-Kt}$, where C is the hydrogen concentration at time t, and C_0 the initial concentration. The flow constant, K = F/λ, where F = blood flow, expressed in ml/min/100g tissue and λ is the partition coefficient of hydrogen in bone relative to that in blood[23]. We calculated the value of λ to be 0.72 for rat cortical diaphyseal bone in a previous experiment (author's unpublished data), as it had not been determined for this species previously.

Capillary density

Before euthanasia, the aorta distal to the renal arteries was cannulated and flushed with 60 mL of heparinized saline under physiologic pressure. After euthanasia, Microfil (Flow Tech, Carver, MA), a colored synthetic angiographic polymer, was infused through the aorta under physiologic pressure. AV bundle patency was again assessed. The compound was allowed to polymerize for 24 hours at 4°C. The bone was then fixed in 10% formalin for 48 hours and decalcified in 14% ethylenediaminetetraacetic acid (EDTA) for 7 hours in a calibrated laboratory microwave at 750 W (Pelco Biowave 3450 Laboratory Microwave, Ted Pella, Inc., Redding, CA). The intraosseous vasculature was visualized by modified Spalteholz methylsalicylate optical bone clearing[25,26] and the capillary density (*D*) of each specimen was measured using

image analysis software (Scion Image; Scion Corporation, Frederick, MD): *D* = vessel pixels/total pixels.

Histological analysis

After decalcification, proximal and distal samples were cut transversely and embedded in paraffin. Paraffin sections were stained with hematoxylin-eosin (H&E). In the transverse section, the number of osteocyte-occupied lacunae and empty lacunae were counted in three fields (400× magnification), which were selected out of cortical bone at random. As an assessment of transplanted bone viability, the percentage of viable osteocytes /total lacunae × 100 was used. Lacunae that were entirely vacant or occupied by a cell with a clearly pycnotic nucleus were considered as nonviable.

Statistical Analysis

Data were expressed as mean ± standard error (SE), and statistical significance was determined using a Kruskal-Wallis test in overall comparisons for each factor of interest. A Wilcoxon rank sum test was used for pairwise comparisons between categories of rats. The Bonferroni adjustment for multiple comparisons was used in these pairwise comparisons. A value of $\alpha < 0.05$ was considered statistically significant.

Results

Patency of AV bundle /microsurgical anastomosis

The AV bundle remained patent until sacrifice in 41.9% (26 of 62 rats) overall. Tacrolimus immunosuppression improved this result with 75.8% (25 of 33) remaining patent at 2 and 4 weeks. Two week animals treated with FK506 had significantly higher AV bundle patency rates than non-immunosuppressed animals ($p < 0.05$). In the non-immunosuppressed rats, none of the microsurgical anastomoses were patent at 2 weeks. At 4 weeks survival, the anastomoses remained open in 14 of 29 rats (51.7%), two weeks after withdrawal of immunosuppression.

Bone blood flow and capillary density

The effect of nutrient artery and AV bundle patency on blood flow and capillary density was evaluated based upon 4 remaining animals in group I, 11 each in groups II, III, IV and V, and 10 in group VI. Animals were excluded from analysis due to perioperative death, wound healing problems and unexpected thrombosis of patent AV bundles.

In the non-immunosuppressed rats, bone blood flow was very low at 2 weeks in all groups (Figure 5). Differences between groups were not significant. Capillary density, however, was significantly greater with AV bundle implantation than in ligated bundle controls ($p < 0.05$). We could not demonstrate a significant beneficial effect of FK506 immunosuppression on bone blood flow or capillary density at 2 weeks. In 4 week groups, both bone blood flow and capillary density were greatest when both the AV bundle and the microvascular pedicle remained patent. This was dependent upon the use of short-term FK506. When only one vessel, (either AV bundle or pedicle) remained patent, blood flow demonstrated intermediate values (Figure 6). No flow or neoangiogenesis were seen when both AV bundle and pedicle were no longer patent.

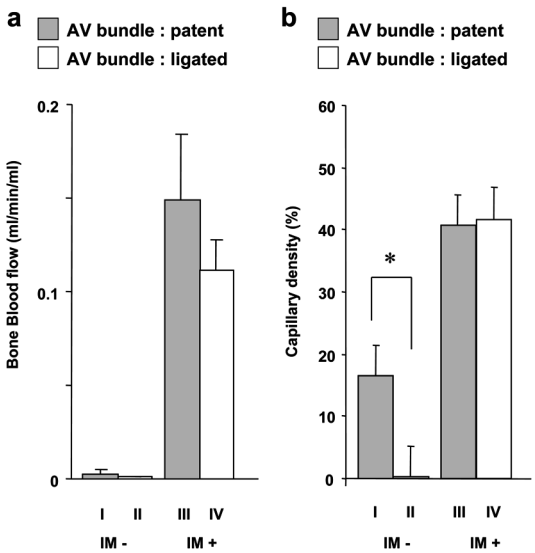

Figure 5. Bone blood flow and capillary density in the 2 week groups (Groups I, II, III and IV). Bars represent means ± SE. *$p < 0.05$.

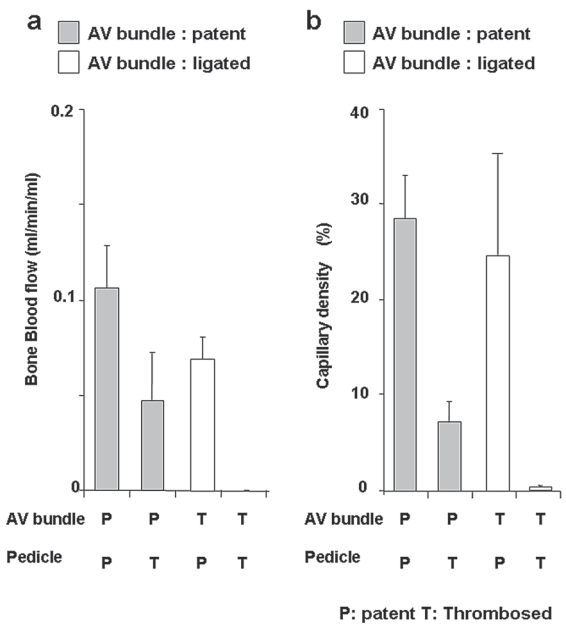

Figure 6. Bone blood flow and capillary density in the 4 week groups (Groups V and VI). Bars represent means ± SE.

Angiography

Neovascularization in the medullary canal was observed along the implanted AV bundle in the groups with patent AV bundles. No neovascular invasion into cortical bone was observed at two weeks, but was evident at 4 weeks (Figure 7).

Histology and osteocyte viability

Rats with patent AV bundles had a high level of viable osteocytes at 4 weeks. All of these animals received FK506 for the first 2 weeks postoperatively. The inner layer cortex, however, was composed of empty lacunae, invaded by neoangiogenic tissue (Figure 8a-c). Although fluorochrome labeling was not performed, evidence of viable osteocytes was observed in the lacunae of cortical bone. In contrast, non-immunosuppressed, AV bundle-patent animals had no significant neoangiogenesis, and necrotic cortical bone at two weeks (Figure 8d-f). The percentage of viable osteocytes was not significantly affected by AV bundle patency at 2 weeks (Figure 9). In the 4 week groups, a significant positive effect of a patent AV bundle on osteocyte viability was seen.

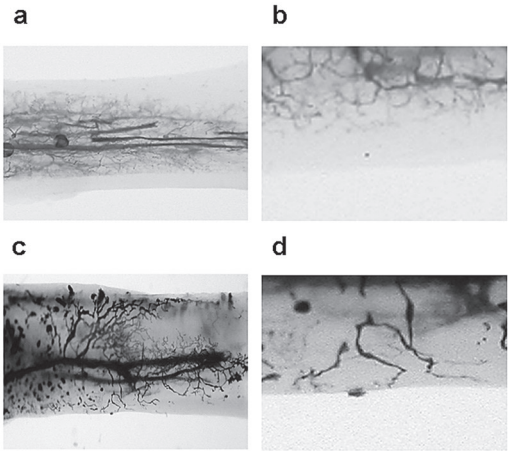

Figure 7. Microangiography in Group I (a, b) and in Group V (c, d). In the medullary canal, (a, c) a patent AV bundle was observed. Under high magnification (×400), no AV bundle neoangiogenesis was observed in the cortical bone of Group I (b), but was evident in the cortical bone of Group V (d).

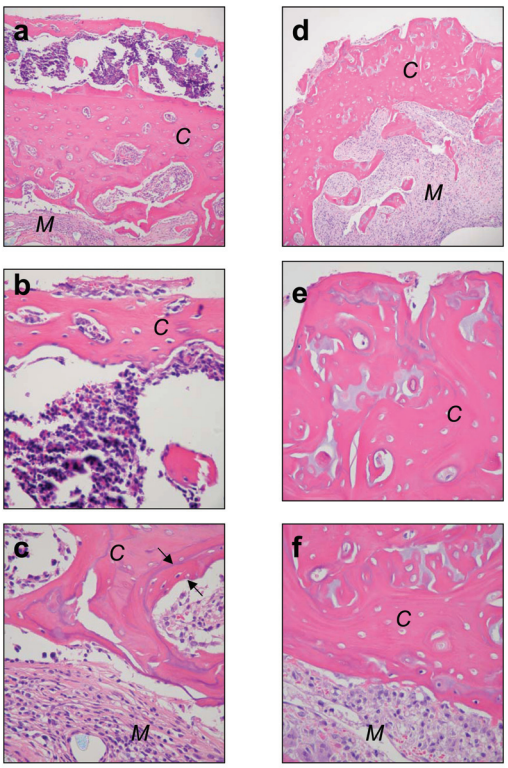

Figure 8. Histology (H & E staining) of the 4 week group with a patent AV bundle (a-c) and in the 2 week group with a patent AV bundle (d-f). Low magnification at ×100 showing cortical (C) and medullary (*M*) bone (a, d). In the 4 week group with a patent AV bundle (b), the outer layer is comprised of lacunae with osteocytes. At ×400, the inner layer cortex (c), while composed of empty lacunae, was invaded by new vessels, and new bone formation is observed (*between arrows*). In the 2 week group with a patent AV bundle (e, f), the absence of osteocytes was observed (magnification ×400), showing that neoangiogenesis from the AV bundle had not as yet reached the endosteal surface of the cortex.

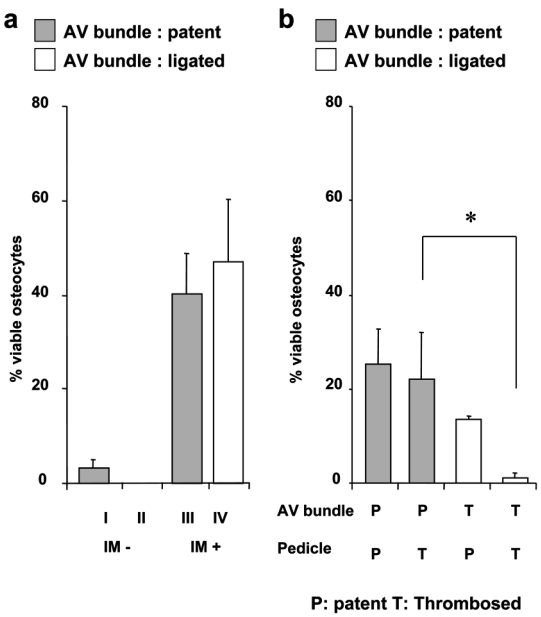

Figure 9. The percentage of viable osteocytes in the 2 week groups (a) and in the 4 week groups (b). In the 4 week groups, a patent AV bundle showed significant osteocyte viability. Bars represent means ± SE. *$p < 0.05$.

Discussion

In the present experimental study we investigated the possibility of maintaining circulation and cellular viability in allotransplanted bone with a combination of microvascular repair of bone nutrient vessels, a short (2-week) course of FK506 immunosuppression, and implantation of a host arteriovenous bundle. We hypothesized that these host AV bundles would develop a neoangiogenic response when implanted into vascularized allogenic femora, maintaining blood flow after withdrawal of short-term immunosuppression. Our results support this hypothesis, demonstrating measurable blood flow in the transplanted bone originating from the implanted AV bundle two weeks after withdrawal of FK506, despite vascular pedicle thrombosis. Although data in this initial short-term study is preliminary, surviving osteocytes were observed in the lacunae of cortical bone.

The patency rate of the AV bundles implanted in the femoral medullary canal was unexpectedly low. Possible explanations for thrombosis could be a low flow state due to an insufficient capillary network between the saphenous artery and vein, and technical issues such as surgical damage to the bundle, compression by the surrounding tissue or hematoma, and kinking of the AV bundle at the entry point into the medullary canal. However, these potential causes do not account for the difference in AV bundle patency rate observed between non-immunosuppressed and FK-506-treated rats. Vascular bundle thrombosis rate was significantly lower in the immunosuppressed rats. This suggests a possible immune mechanism for the observed thrombosis. A difference in AV bundle patency affected by immune mechanisms has been observed by others as well[27]. In this study, implanted AV bundles placed within fresh bone allografts thrombosed at a higher rate than those placed into less immunogenic previously frozen allografts. They also found fresh allografts to rapidly breakdown and fragment in this laboratory rat model of conventional (nonvascularized) bone allotransplantation.

The percentage of viable osteocytes in the vascularized bone recipients with patent AV bundles was significantly higher than in nonvascularized bone transplants, despite the fact that only 40% and 23% of the microsurgical repairs supplying the nutrient pedicles remained patent at 2 and 4 weeks. These patency rates were slightly less than previous similar allogenic limb transplant reports in experimental animals[28-31]. Most of the viable osteocytes were found in the outer cortex, the inner layer being largely necrotic (empty osteocyte lacunae). This can be explained by the medullary canal reaming necessary to allow AV bundle insertion, thus damaging bone endosteal circulation. A two week survival time is insufficient to allow implanted AV bundles to revasculariize the inner layer of cortical bone. At 4 weeks, however, implanted AV bundles did revascularize the endosteal surface, but new bone formation was minimal. As an adequate vascular supply plays a pivotal role in osteogenesis[12], we expect a long-term study using our model would document the ability of implanted AV bundles to both revascularize and remodel necrotic compact bone. Further research with long-term survival is planned.

Unlike other organ transplants, living allogeneic bone has the unique property of maintaining its function to provide structural support for a considerable period of time after rejection. Indeed, nonvascularized or conventional bone structural

allografts may gradually weaken and fracture, while living bone autotransplants maintain strength and hypertrophy in response to stress. While one might expect living bone allotransplants to behave similarly as long as organ rejection is prevented by adequate systemic immunosuppression, significant osteopenia has been demonstrated to result from such treatment in experimental bone allotransplants[32]. The sequelae and potential complications of life-long systemic immunosuppression are generally viewed as unacceptable for non-life-critical tissue transplants such as bone, however. Ikeda documented that transplanted allograft bone, once replaced by new bone derived from the recipient will exhibit increased strength, even with incomplete replacement[33]. During revascularization, however, bone strength diminishes, requiring additional protection in the form of hardware, casts or braces for an extended period of time. Maintaining viability of the transplanted bone as a supportive organ is very important.

Conclusions

We have sought in this study to develop a method that may allow bone survival without long-term immunosuppression or tolerance induction. Transplanted allogeneic bone, combined with an implanted host AV bundle may be able to maintain bone nutrition, structure and strength without long-term strong immunosuppression. Furthermore, the implanted bundle may accelerate the process of repopulation of the organ by living, host-derived tissue. Previous work from our laboratory has in fact demonstrated more than 90% of total cells in vascularized rat tibial allografts to be replaced by cells of recipient origin[34-36]. This study demonstrates that vascularized bone allografts maintain blood flow and viability in the short term, even after termination of immunosuppression. Long-term survival studies will be needed to explore extent of neoangiogenesis, demonstrate new bone formation and cellular lineage of surviving osteocytes, and measure the strength of orthotopically-placed experimental bone allotransplants before clinical application can be considered.

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3

SHORT-TERM IMMUNOSUPPRESSION AND SURGICAL NEOANGIOGENESIS WITH HOST VESSELS MAINTAINS LONG-TERM VIABILITY OF VASCULARIZED BONE ALLOTRANSPLANTS

Introduction

We explored the use of our model of implantation of host-derived vessels together with primary anastomosis of donor blood vessels in a short-term study (Chapter 2), and found promising results. In this chapter, we will show that these results are maintained in the long term. This approach may allow functional reconstruction of complex musculoskeletal tissues, including entire joints with adjacent bone, without the complications associated with long-term immunosuppression.

Methods

Experimental Design

Vascularized femoral allotransplantation was performed across a MHC barrier. Sex-mismatched (female to male) transplantation was performed to aid in later identification of cellular lineage within the transplanted tissue. In all recipient animals, the allogeneic femur was transplanted to an abdominal wall pocket, as described in the previous chapter, with microvascular restoration of the nutrient vessel circulation. Rats were then randomly allocated to one of 4 groups, which differed in the use of immunosuppression and patency of the implanted host AV bundle (Table 1). Experiments were terminated at 18 weeks. Blood flow to the allotransplanted femurs was measured with the hydrogen washout technique, and then the arterial system was injected with a colored silicone rubber injection compound. Portions of the bone were further analyzed by Spalteholz clearing and image analysis for capillary density and histologic evidence of rejection and osteocyte viability (osteocyte count).

Table 1: Experimental Groups

Group	AV-Bundle	Immunosuppression	n
1	Patent	None	11
2	Ligated	None	7
3	Patent	FK-506, 2 weeks	11
4	Ligated	FK-506, 2 weeks	9

Animals and anesthesia

The surgical model and anesthesia employed was identical to that described in Chapter 2. Eighty six inbred female Dark Agouti rats (DA, genetic expression: RT1^a)

weighing 150-175 g were used as donors and eighty six inbred male Piebald Virol Glaxo rats (PVG, genetic expression: RT1^c) weighting 245 ± 32 g were used as recipients (Harlan Sprague Dawley, Madison, WI). All experiments were performed according to established NIH guidelines under the direction of the Institutional Animal Care and Use Committee.

Aftercare and exclusion criteria

Immunosuppression sufficient to maintain short-term graft viability and nutrient vessel patency was provided by FK-506 (Tacrolimus, Fujisawa Pharmaceutical Co., Osaka, Japan), 1mg/kg/day for 14 days in Groups 3 and 4[1]. Rats were allowed to move freely postoperatively. Animals were inspected daily, and wound care administered as necessary. Unresolved wound healing problems lead to early euthanasia and exclusion from the study in 33 of 86 rats. An additional 6 animals were excluded based upon premature death and another 9 animals excluded due to thrombosed AV bundles in the patent AV bundle Groups 1 and 3. This left 38 animals remaining for evaluation.

Evaluating and harvesting grafts at termination of experiment

Eighteen weeks after the initial transplantation, bone blood flow by hydrogen washout and capillary density measurements were performed as described in Chapter 2.

Histologic grading of bone viability

The decalcified paraffin-embedded proximal and distal segments of bone specimens were sectioned and stained with hematoxylin-eosin. Bone viability and rejection was evaluated by light microscopy. Two sections of bone were graded for each animal and the average score used. Grading was done on a four-point scale with *Grade 0* having no bone necrosis and normal histology; *Grade 1*, mild bone necrosis with less than half of the osteocytes having partially empty lacunae and decreased peritrabecular osteoblast lining; *Grade 2,* moderate bone necrosis with more than half of the osteocytes having partially empty lacunae and no peritrabecular osteoblast lining; *Grade 3,* severe bone necrosis with osteocytes having completely empty lacunae and fragmentation.

Statistics

Experimental animal characteristics are reported as median (minimum, maximum) for continuous variables. Capillary density, blood flow, and histology were compared between all four groups using the Kruskal-Wallis test, and the Wilcoxon Rank Sum test was utilized for the pairwise comparisons. The p-values are not adjusted for multiple comparisons. Analyses were performed with SAS v8.2 (SAS Institute Inc, Cary, NC). All tests were two sided, and p-values less than 0.05 were considered statistically significant.

Results

Nutrient pedicle and AV bundle patency

The efficacy of host AV bundle implantation in maintaining tissue viability was evaluated based on AV bundle and nutrient pedicle patency, measurement of bone blood flow, quantification of the extent of neoangiogenesis from the AV bundle, and determination of osteocyte viability. Direct inspection of the vascular pedicle (bone nutrient circulation) was difficult, as all pedicles were enveloped in friable granulation tissue from the site of microvascular anastomoses distally. Dissection through this tissue would have carried substantial risk of vessel injury. The implanted host-derived AV bundles were also surrounded by reactive tissue, although less so than the allograft vessels. *Patency* was therefore evaluated by identification of each vessel with microangiography. The control groups with ligated bundles (Groups 2 and 4) had no identifiable AV bundle as expected. Transparent bone specimens contained a longitudinally-oriented intramedullary vascular bundle in those groups with a patent AV bundle (Groups 1 and 3). Seven of 18 (39%) non-immunosuppressed Group 1 animals and 2 of 13 (15%) immunosuppressed Group 3 animals had thrombosed AV bundles based upon this assessment. This apparent difference was not statistically significant (p=0.24). None of the nutrient vessels were patent at 18 weeks, probably due to rejection following withdrawal of FK-506. Subsequent statistical analysis of the effect of implanted host AV bundles on capillary density, bone blood flow and osteocyte viability included only those animals with demonstrably patent AV bundles from Groups 1 and 3.

Cortical bone blood flow

Bone blood flow measurements were obtained from the diaphyseal cortex during the final nonsurvival surgery (Figure 1). There was a statistically significant difference in blood flow among the four groups ($p<0.01$). The mean (range) blood flow in animals with patent AV bundles measured 0.00 (0.00 - 0.21) ml/min/ml with no immunosuppression (Group 1) and 0.12 (0.00 - 0.27) ml/min/ml with short-term immunosuppression (Group 3, $p=0.08$). In those animals with ligated AV bundles, mean cortical blood flow measured 0.00 ml/min/ml, with the following ranges: 0.00 - 0.07 ml/min/ml in Group 2 and 0.00 - 0.09 ml/min/ml in Group 4. Cortical bone blood flow in the animals with ligated AV bundles (Groups 2 and 4) was significantly less than in animals with patent AV bundle implantation and transient immunosuppression (Group 3) ($p=0.01$ and <0.01, respectively). Blood flow in femora with patent AV bundles but no immunosuppression (Group 1) was not significantly different than ligated Groups 2 and 4 ($p=0.18$ and $p=0.11$ respectively). Blood flow was not measurable in six of the seven Group 2 animals, and the remaining rat had a blood flow of 0.07 ml/min/ml.

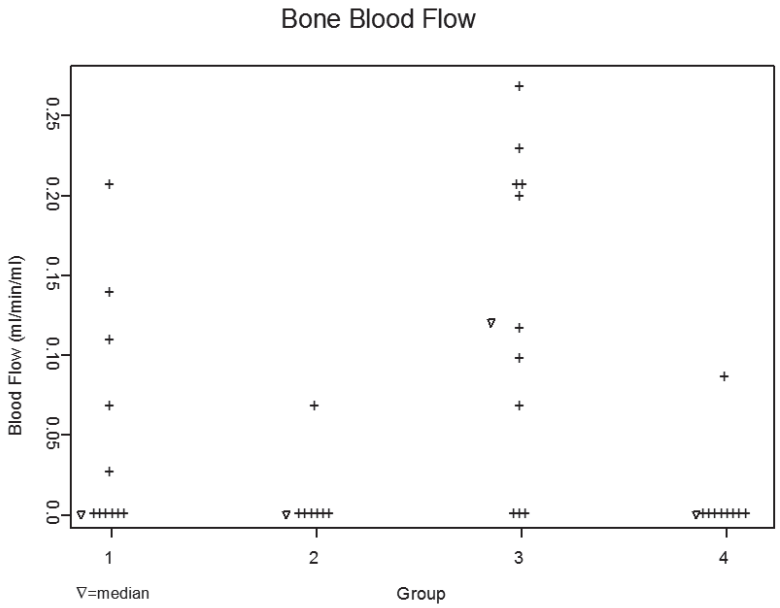

Figure 1. Bone Blood Flow (ml/min/ml) measured using the hydrogen washout technique. Group 1 = Patent AV bundle, no immunosuppression; Group 2 = Ligated AV bundle, no immunosuppression; Group 3 = Patent, FK506 immunosuppression; Group 4 = Ligated AV bundle, FK506 immunosuppression.

Neoangiogenesis (capillary density)

We measured the density of neoangiogenic capillaries after microangiography and bone clearing. The median capillary density was as follows: Group 1, patent AV bundle and no immunosuppression: 10.56% (0 - 26.74%); Group 2, ligated AV bundle and no immunosuppression: 0.27% (0 - 11.82%); Group 3, patent AV bundle and FK-506 immunosuppression: 23.49% (8.01 - 56.70%); Group 4, ligated AV bundle and FK-506 immunosuppression: 6.46% (0 - 28.16%). There was a statistically significant difference in capillary densities among the four groups ($p<0.01$). Pairwise comparisons demonstrated Group 3 (patent AV bundle + short-term immunosuppression) vessel density to be higher than Groups 1, 2, or 4 ($p<0.01$, $p<0.01$, and $p<0.01$, respectively) (Figure 2). Group 1 was also significantly higher than Group 2 ($p=0.04$). In the non-immunosuppressed animals we often saw sparse neovascularization from patent AV-bundles as compared to robust capillary formation from patent AV bundles in immunosuppressed animals (Figure 3).

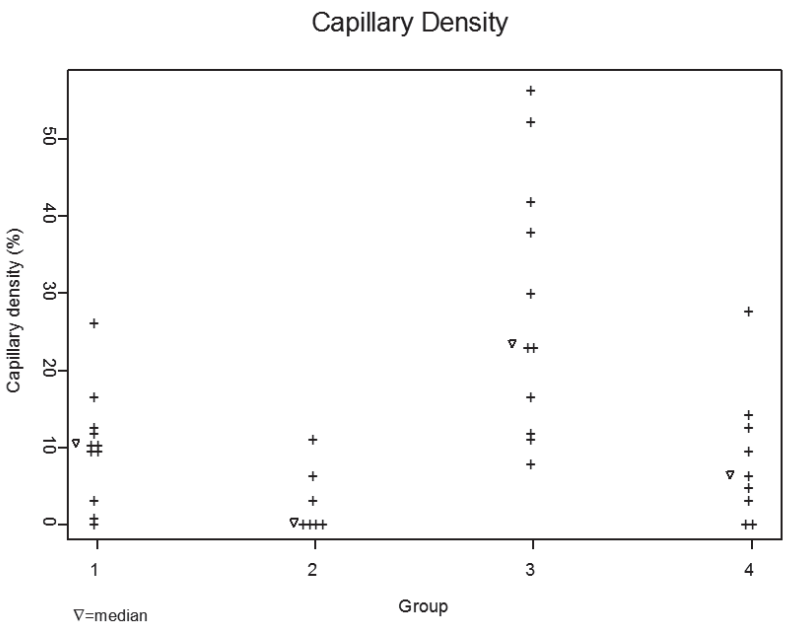

Figure 2. Capillary Density. Neoangiogenesis from implanted AV bundles was quantified by capillary density expressed as the percentage of the total bone area occupied by vessel. Group 1=Patent AV bundle, no immunosuppression; Group 2=Ligated AV bundle, no immunosuppression; Group 3 = Patent AV bundle, FK506 immunosuppression; Group 4= Ligated AV bundle, FK506 immunosuppression.

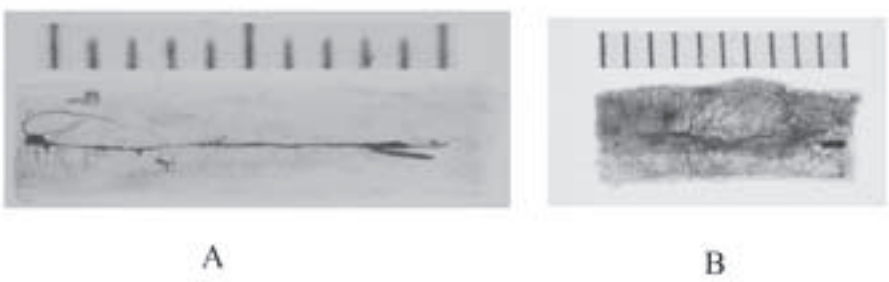

Figure 3. Microangiography. A) Low rate of neovascularization as seen in Group 1 results (patent AV-bundle, no immunosuppression). B) High rate of neovascularization as seen in Group 3 results (patent AV-bundle, 2-week FK506 immunosuppression).

Bone viability/necrosis

Bone necrosis, based upon osteocyte counts, was graded on a 4 point scale, from a normal value of zero (no osteocyte necrosis) to four (all lacunae empty). Two measurements per specimen were averaged (Figure 4). Median values were as follows: Group 1 (patent AV bundle and no immunosuppression), 2.5; Group 2 (ligated AV bundle and no immunosuppression), 3; Group 3 (patent AV bundle and FK-506 immunosuppression), 1.5; and Group 4 (ligated and FK506 immunosuppression), 2.5. There was a statistically significant difference in osteocyte viability among the four groups ($p<0.01$), with all significant pairwise comparisons showing Group 3 to have better results than Groups 1, 2, or 4 ($p<0.01$, $p<0.01$, and $p=0.02$, respectively). Group 2 had poorer viability, significantly different than Group 1 or 4 ($p=0.03$, $p<0.01$ respectively). Bone viability was best when short term immunosuppression was supplemented by patent AV-bundle implantation (Group 3).

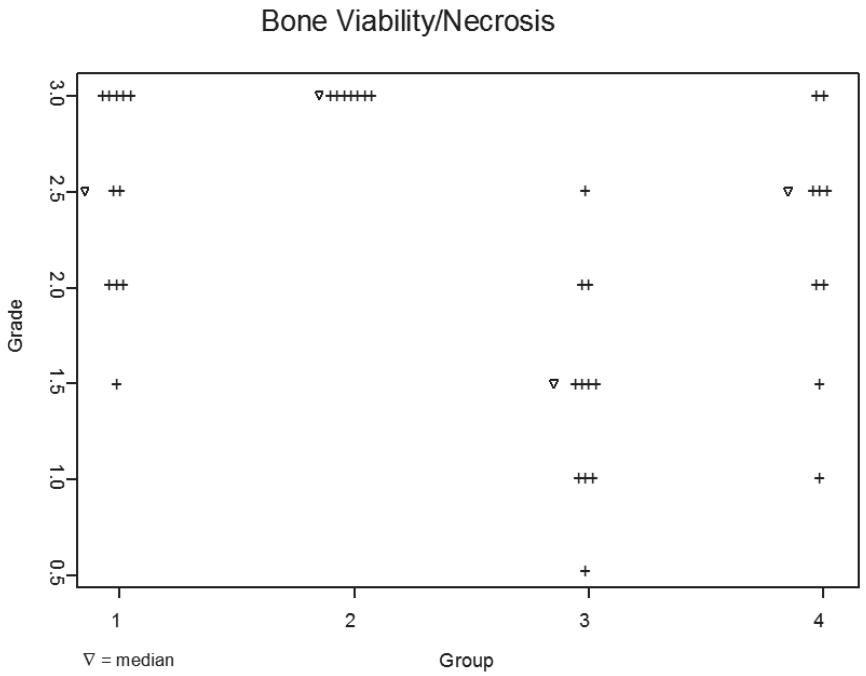

Figure 4. Histological grading of bone viability/necrosis. Bone necrosis was least severe in Group 3 specimens (with patent AV-bundle and FK506 immunosuppression).

Discussion

There is considerable clinical potential for transplantation of living musculoskeletal tissues given the shortcomings of other available methods. For example, conventional (nonviable) allografts are prone to non-union[2] and late stress fracture[3]. Vascularized autograft sources are limited in size and shape, and prosthetic replacement of large structural defects fail at a high rate for a variety of reasons[4,5]. Accordingly, few vascularized bone allotransplant procedures have been performed[6,7]. Risks associated with immunosuppression or tolerance induction, permissible for life-critical organ transplantation and required at present for their survival, are difficult to justify for routine reconstructive surgery of the extremities, however.

In this study, a novel combination of surgical angiogenesis from host vessels and *short-term* immunosuppression was used to maintain vascularized bone allotransplant blood flow and viability. Immunosuppression was administered only for two weeks, sufficient for angiogenesis from the host-derived vessels to occur. This approach allowed blood flow and tissue viability to be monitored despite vascular rejection of the original microvascular surgical repair.

Angiogenesis is a biologic process of new capillary formation. Surgical transfer of vessels or well-vascularized tissue into avascular bone induces neoangiogenesis and improves blood flow[8]. We previously found that implanted AV bundles can induce angiogenesis and remodelling in bone[9], allotransplants (Chapter 2)[10] and xenogeneic bone[11]. Kumta et al. demonstrated that vascular bundle implantation into fresh nonvascularized allografts resulted in an aggressive inflammatory response and an increased rate of spontaneous AV bundle thrombosis when compared to less immunogenic frozen allografts[12]. While other reasons for AV bundle thrombosis, such as a paucity of flow in the ligated pedicle or kinking of the vessels play a role in such events, our data supports an immune mechanism as a prominent factor. Non-immunosuppressed groups had consistently higher rates of thrombosis than those whose animals received short-term immunosuppression in this study (33 vs. 15 %, respectively). Neoangiogenesis from the host-derived AV bundle also occurred most robustly in animals that received short-term immunosuppression.

We have recently demonstrated that bone allotransplant cells are gradually replaced with host-derived cells over time, using a semi-quantitative polymerase chain reaction analysis of a Y-chromosome-specific marker after sex-mismatched

transplantation of bone across a major histocompatibility barrier with continuous immunosuppression[13]. Quantitative analysis of osteocyte lineage using laser capture microdissection is the subject of Chapter 8[14].

In this study, a patent AV bundle enhanced by short-term FK-506 maintained a significant level of osteocyte viability, although areas of necrotic bone could be identified. No nutrient vessel repairs remained patent at the termination of the study presumably due to immune mediated rejection and trombosis. The ability of neoangiogenic capillaries to supply nutrition to the bone sufficiently to maintain weight-bearing function, remodeling and healing requirements remains to be demonstrated in a future study.

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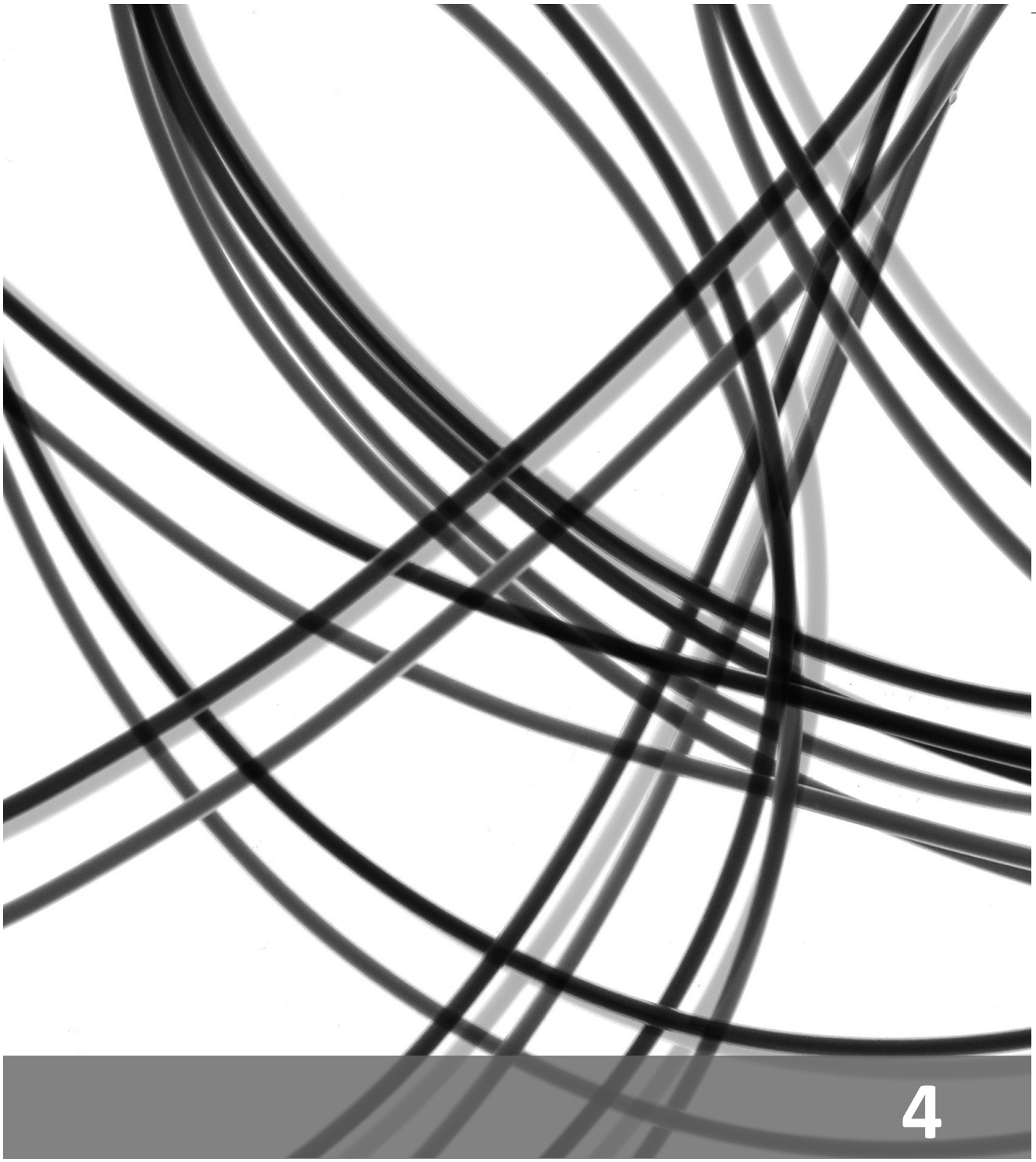

4

LIVING BONE ALLOTRANSPLANTS SURVIVE BY SURGICAL ANGIOGENESIS ALONE: VALIDATION OF A NOVEL METHOD OF COMPOSITE TISSUE ALLOTRANSPLANTATION

Introduction

In the experiment described in this chapter, we quantify blood flow and extent of angiogenesis, demonstrate biologic activity through new bone formation, and assess whether a state of immune tolerance could otherwise account for maintained tissue viability. This builds on the results from previous experiments described in Chapters 2 and 3, mainly by proving that a state of immune tolerance is not needed for our model to be successful. We also assess the model in comparison to non-vascularized allografts and vascularized isotransplants. When applied clinically, these methods may eventually provide the ability to replace missing bone with living tissue of similar size and shape, while maintaining the superior healing and remodeling abilities of living bone noted in vascularized autogenous bone grafts.

Materials and Methods

Experimental design and operative procedure

The experimental groups are shown in Table 1. Inbred female Dark Agouti rats (DA) weighing 150-175 g were used as femoral allotransplant donors in Groups 1 and 2, and as allograft donors in Group 3. For the isotransplant controls (Group 4), we used female Piebald Virol Glaxo rats (PVG) weighing 200 to 250 g. Male PVG rats weighing 200 to 250 g were used as the recipient animals. All animals were obtained from the same supplier (Harlan Sprague Dawley, Madison, WI). Groups 1 and 2 differed only in the patency of the implanted AV bundle. Paired comparison between groups allowed evaluation of AV bundle effect in allotransplanted femora (ligated in Group 2 versus patent in Group 1), bone allogenicity (isogeneic in Group 4 rather than allogeneic Group 1 transplants), and initial tissue circulation/viability (Group 1 allotransplant versus Group 3 allograft). All experiments were performed according to established National Institutes of Health guidelines and under the direction of our Institutional Animal Care and Use Committee.

The operative procedure and bone blood flow and capillary density measurement procedures were identical to those described in Chapter 2. In Groups 1, 2 and 4 vascularized femoral allo- or isotransplants were harvested as there described, while frozen allografts (Group 3) were harvested without vascular pedicles. In that Group, all soft tissue was removed from the allograft, which after irrigation was frozen in

liquid nitrogen without cryoprotectants for 30 minutes, followed by storage at -70°C for a minimum of 24 hours. In Group 3, the previously-frozen femoral allograft was also positioned into the abdominal pocket but without microvascular repairs.
In a second survival procedure 18 weeks after initial surgery, three 15x15 mm full-thickness skin defects were made on the back of each recipient. Full-thickness skin grafts from donor-, recipient- and third-party-type (Lewis, LEW) rats were transplanted to each of the defects and fixed with a bolster dressing for five days. Bone blood flow measurements and graft harvest were performed after 21 weeks.

Grading of skin graft acceptance

Skin graft acceptance was graded on the extent to which DA, PVG and LEW grafts were viable at experiment termination: 0-69% (poor acceptance), 70-85% (fair acceptance), 86-100% (good acceptance). Our grading system corresponds with clinical assessments.

Table 1 (Experimental Groups). Independent variables were a/v bundle patency, allotransplant viability and antigenicity. Immunosuppression: FK-506, 1 mg/kg/day.

Group	Number	Group Descriptions	Immuno-Suppression	A/V Bundle	Survival (weeks)
1	35	Allotransplant: DA female to PVG male	2 weeks	Patent	21
2	28	Allotransplant: DA female to PVG male	2 weeks	Ligated	21
3	11	Allograft: DA female to PVG male	2 weeks	Patent	21
4	19	Isotransplant: PVG female to PVG male	2 weeks	Patent	21

Histologic grading of rejection

Rejection was graded (none = 0, mild = 1, moderate = 2, or severe = 3) on hematoxylin-eosin stained specimens based on the amount of inflammation at 400X magnification, and performed for a section of transplant pedicle and bone medullary canal[1]. To measure bone viability, the number of osteocyte-occupied lacunae versus empty lacunae were counted in three randomly selected fields (400X magnification).

The results were graded: *Grade 0*, normal histology; *Grade 1*, less than half empty lacunae and decreased peritrabecular osteoblast lining; *Grade 2*, more than half empty lacunae and no peritrabecular osteoblast lining; *Grade 3*, severe necrosis with empty lacunae and fragmentation.

Quantitative histomorphomtery

Calcein and tetracycline fluorescent markers were injected at the base of the tail vein at a dose of 20 mg/kg at 2 weeks and 2 days prior to sacrifice, respectively. Glycol methylmethacrylate-embedded 5 µm-thick transverse sections were assessed using quantitative bone histomorphometry software (Osteomeasure®; Osteometrics, Atlanta, GA). Ten randomly selected fields at 200X magnification were studied. Perimeters of trabecular bone surface, osteoblast-covered surface, eroded surface, and osteoclast-covered surface were measured. From three random samples from each group, adjacent transverse sections were stained by modified Goldner's trichrome stain for mineral deposition and analyzed at 20X and 400X magnification.

Statistical Methods

Experimental characteristics are reported as medians (minimum, maximum) for continuous variables. Since the data were not normally distributed, nonparametric tests were used whenever possible, and all descriptive information was reported as n (%) or median (range). Histology grading and skin graft acceptance scores were compared overall and for each combination of two groups using a Cohran-Mantel-Haenszel extension of Fisher's exact test for ordered contingency tables[2]. AV bundle status was compared with capillary density and blood flow overall using a Kruskal-Wallis test, and for each combination of two groups using the Wilcoxon rank sum test. The femoral medullary canal and cortex were analyzed histologically using 10 random areas from 10 different sections of 10 specimens by two independent reviewers. All statistical tests were two-sided and p-values less than 0.05 were considered statistically significant. Analyses were performed with SAS v9.1 (SAS Institute Inc, Cary, NC).

Results

Significant differences between groups were found for blood flow, capillary density and bone formation rates ($p \leq 0.003$, Kruskal-Wallis test), as well as in bone viability grade and medullary canal inflammation ($p < 0.001$, Cochran-Mantel-Haenszel test). An overview of results is provided in Table 2.

Table 2 (Results). All values are expressed as medians; 0 = none, 1 = mild, 2 = moderate, 3 = severe for inflammation and rejection.

Group	Blood flow (ml/min/100g)	Capillary Density (%)	Bone formation rate ($\mu m^3/\mu m^2$/year)	Medullary Canal Inflammation	Bone Rejection Grade
1	4.58	15.0	6.4	1.0	1.0
2	0.00	4.4	0.0	1.5	3.0
3	0.00	8.1	24.1	3.0	3.0
4	0.44	16.6	50.3	1.0	0.0

The median bone blood flow was 4.58 ml/min/100g in allotransplants with a patent AV bundle (Group 1). This was significantly higher than isotransplants (Group 4, 0.44 ml/min/100g), allotransplants with ligated AV bundles (Group 2, no flow) or frozen allograft bone (Group 3, no flow) ($p < 0.001$ for each two sample Wilcoxon rank sum test) (Figure 1).

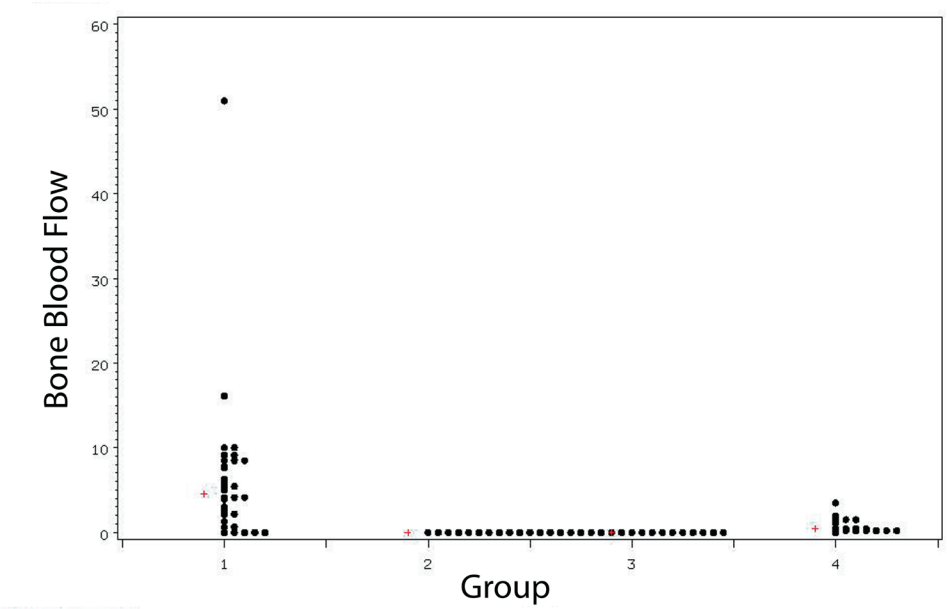

Figure 1. Bone blood flow (ml/min/100g) per group.

In Group 1, bone viability was grade 0 (normal) in 8.8%, grade 1 in 52.9%, grade 2 in 32.4% and grade 3 (severe necrosis) in 5.9% (Figure 2). The respective results for Group 2 were 0.0%, 3.6%, 25.0% and 71.4% (Figure 3). Purely grade 3 histology was found in Group 3. Group 4 bones had either normal histology (57.9%) or grade 1 necrosis (42.1%). Group 1 showed greater viability than Groups 2 and 3, and less than Group 4 ($p<0.001$, Cochran-Mantel-Haenszel test) (Figure 4). Groups 2 and 3 did not differ statistically in bone viability ($p=0.06$), but both had significantly fewer viable osteocytes than Group 4 ($p<0.001$).

R1 R2 R3 R4 R5 R6 R7 R8 R9 R10 R11 R12 R13 R14 R15 R16 R17 R18 R19 R20 R21 R22 R23 R24 R25 R26 R27 R28 R29 R30 R31 R32 R33 R34

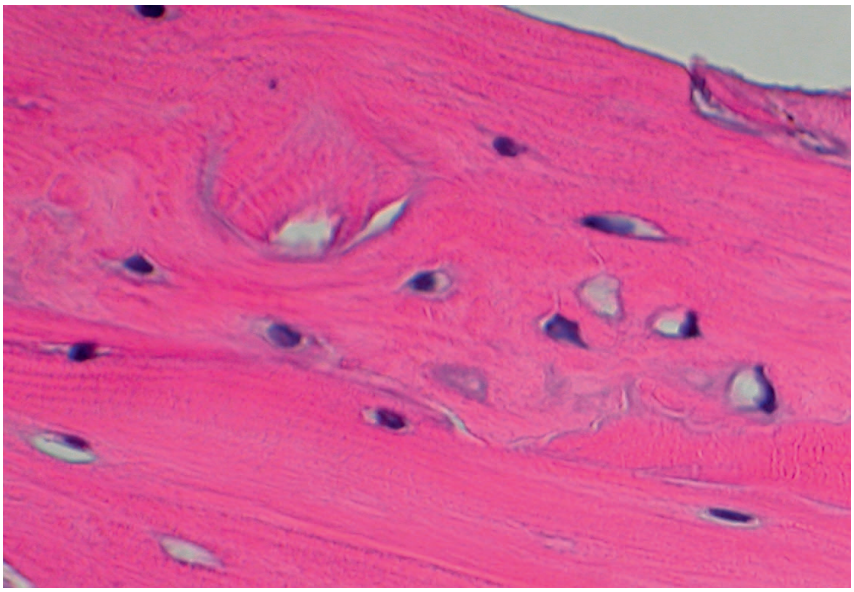

Figure 2. Hematoxylin-eosin section (400X) showing a representative sample from Group 1 with more than 50% lacunae filled with normal osteocytes and normal bone morphology.

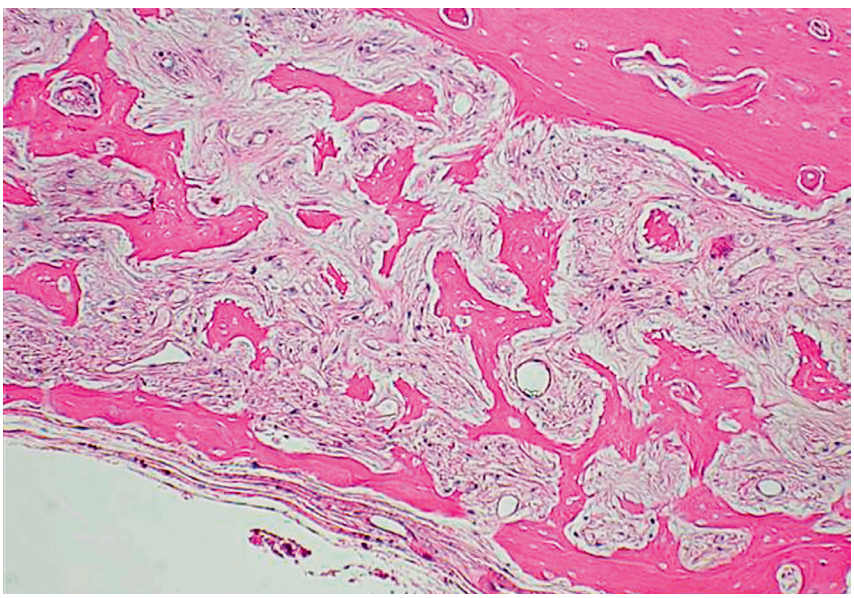

Figure 3. Hematoxylin-eosin section (400X) showing a representative sample from Group 2 with very few osteocyte-holding lacunae, grossly altered bone morphology with cortical necrosis and absent peritrabecular lining.

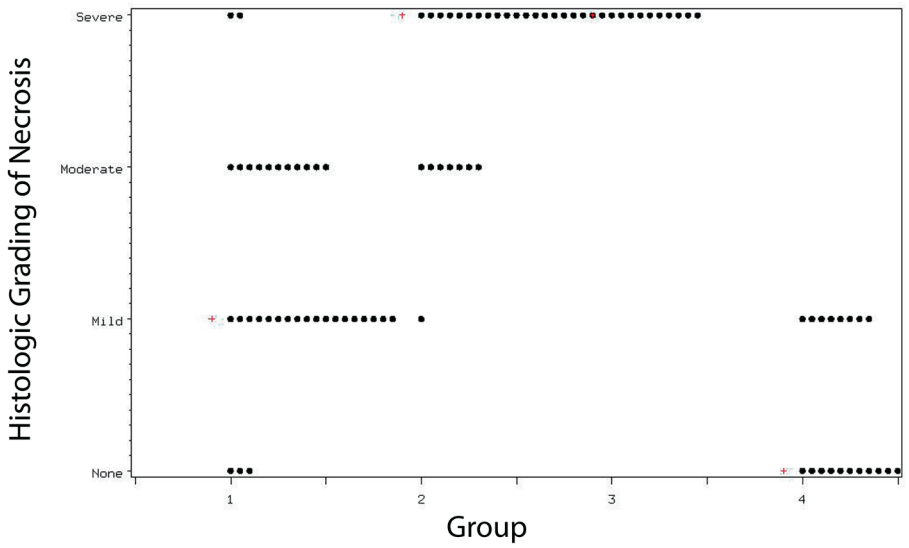

Figure 4. Bone viability per group as measured by histological grading.

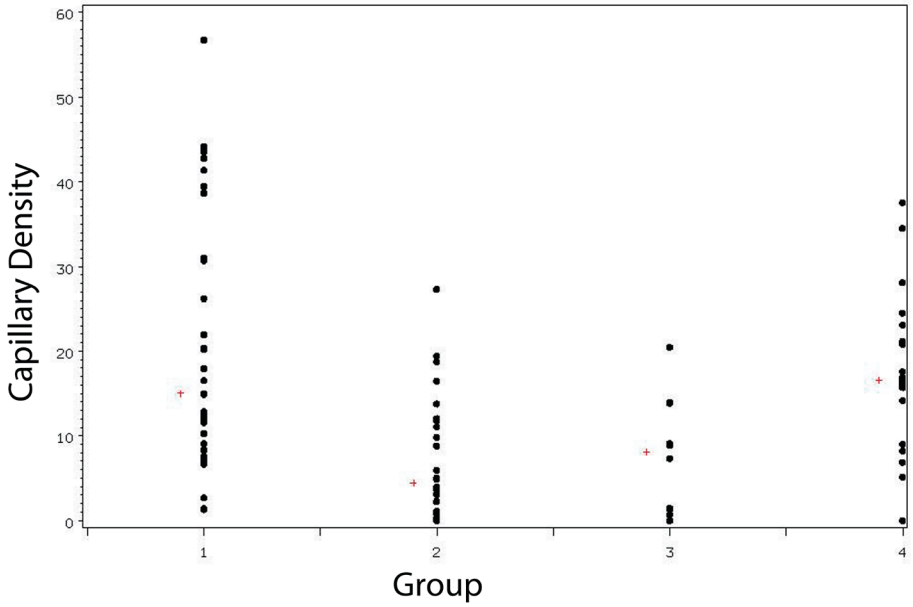

Figure 5. Capillary density (%) per group.

R1
R2
R3
R4
R5
R6
R7
R8
R9
R10
R11
R12
R13
R14
R15
R16
R17
R18
R19
R20
R21
R22
R23
R24
R25
R26
R27
R28
R29
R30
R31
R32
R33
R34

The median capillary density in allotransplant Group 1 was 15.0%, compared to 4.4% in Group 2 allotransplants with a ligated AV bundle (p<0.001). Neoangiogenesis occurred within the allografts to a lesser extent, at 8.1% (p=0.01). Isotransplants (Group 4) had the highest levels, 16.6% (p=0.96) (Wilcoxon rank sum tests) (Figure 5). Figure 6 shows the difference in capillary density in a fully revascularized allotransplant (Group 1) compared to those from Group 2. Groups 2 and 3 did not differ in capillary density (p=0.83), but had less than Group 4 (p<0.001 and p=0.008, respectively).

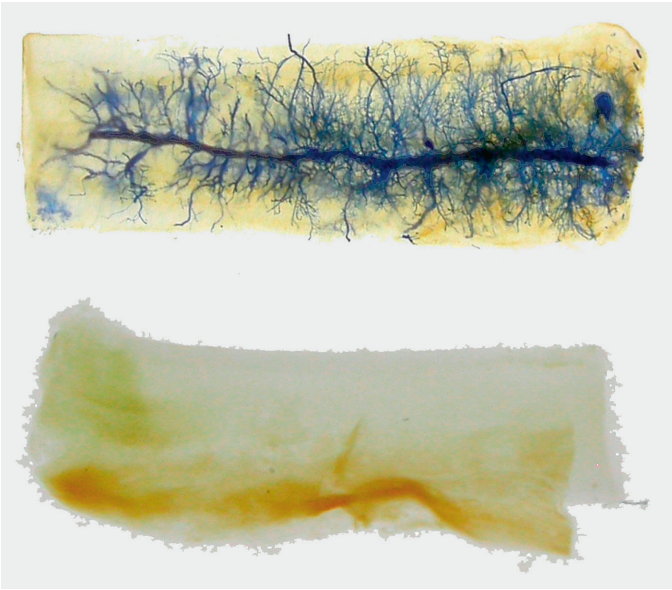

Figure 6. Cropped images showing a representative decalcified and cleared allotransplant from Group 1 (top) and Group 2 (bottom). Polymerized Microfil® visualizes extensive neovascularization from the implanted host-derived AV bundle.

The median bone formation rate per bone surface area (BFRBS, $\mu m^3/\mu m^2$/year) in Group 1 was 6.4, 0 in Group 2 (p=0.002), 24.1 in Group 3 (p=0.097), and 50.3 in Group 4 (p=0.63) (Wilcoxon rank sum tests) (Figure 7). Group 2 had significantly less bone formation than Groups 3 and 4 (p<0.001 and p<0.01, respectively). Groups 3 and 4 did not differ in bone formation rates (p=0.81). Figures 8a and 8b show representative images from Group 1 samples with dual layer calcein and tetracycline fluorescence.

In Group 1, inflammation in the medullary canal was absent in 26.5%, mild in 50.0%, moderate in 20.6% and severe in 2.9%. The respective values for Group 2 were 17.9%, 32.1%, 28.6% and 21.4%, which were significantly greater on comparison ($p=0.04$). Significantly more inflammation was seen in Group 3 than in Groups 1, 2 and 4 ($p<0.001$). Group 1 compared favorably ($p=0.16$) to Group 4, which had inflammation rates of 36.8% (none), 57.9% (mild), 5.3% (moderate), and no severe inflammation (Figure 9).

All DA skin grafts were fully rejected, demonstrating that donor-specific tolerance had not developed. PVG to PVG skin grafts showed good acceptance overall (84% good, 14% fair and 2% poor) demonstrating successful skin grafting technique. LEW to PVG skin grafts were all fully rejected, demonstrating a healthy immune system in the graft recipients.

The percentage of AV bundles that remained patent at the end of the study from among those that were initially patent was 75%. Trichrome stained sections showed clear evidence of osteoblasts and osteoclasts adjacent to and osteocytes inside the mineral bone, with new bone formation around the AV bundle and at the perimeter of the bone. This was clearly more evident in the samples from Groups 1 and 4.

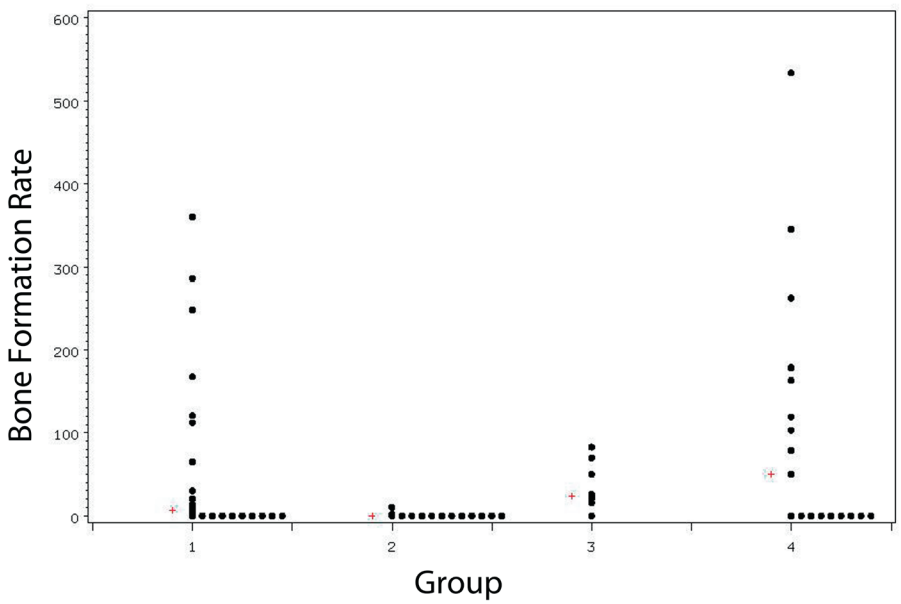

Figure 7. Bone formation rate per bone surface area (µm3/µm2/year) per group.

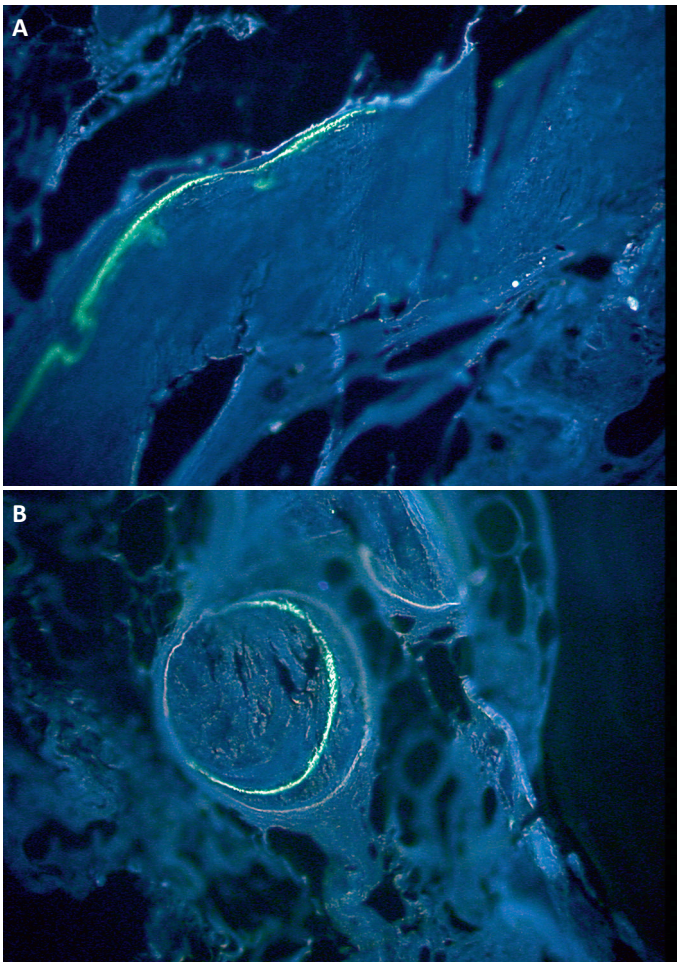

Figure 8. Calcein and tetracycline labeled, methylmethacrylate embedded 5 µm sections at 200X magnification for histomorphometric analysis. Both samples are from Group 1. Double fluorescence was discernible both (a) on the perimeter of the transplant and (b) within the cortical boundaries.

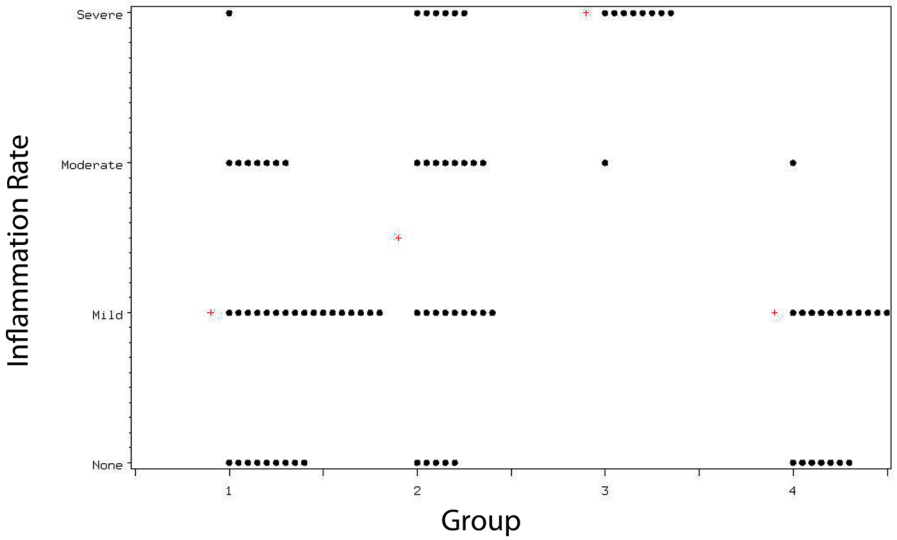

Figure 9. Inflammation rate in the medullary canal per group.

Discussion

The ideal replacement for large bone defects is one which behaves as the missing bone did: viable, non-immunogenic to the host and providing sound structural support. This might be accomplished best by transplantation rather than the use of structural allografting, if the significant risks of long-term immune modulation can be mitigated. The current study adopts the methods of therapeutic angiogenesis to the CTA transplantation problem, creating a novel method of tissue transplantation that requires neither immune suppression nor a state of tolerance. Instead, we have created a transplant chimera with an autogenous blood supply. This is accomplished by microsurgical transplantation and two weeks of drug immunosuppression to maintain short-term viability while angiogenesis from an autogenous AV bundle is occurring. This ultimately makes the nutrient pedicle superfluous and permits drug-free tissue viability as measured by blood flow, capillary density, osteocyte viability and active bone remodeling. We used four experimental groups to study this problem. We evaluated the effect of the AV bundle (comparing allotransplants with ligated and patent AV bundles), bone allogenicity (revascularized isogeneic versus allogeneic transplants), and initial osteocyte viability (allotransplantation versus allografting).

Therapeutic angiogenesis used to treat or prevent tissue ischemia may be accomplished by surgical transfer of well-vascularized autogenous tissue, used alone or augmented by simultaneous administration of vasculogenic cytokines[3-5]. Surgical angiogenesis in autogenous bone grafts was first demonstrated by implantation of a vascular bundle in a canine model[3]. Implantation of vessels has been shown to induce neovascularization and new bone formation in conventional rabbit and canine autografts[4,6,7], canine nonvascularized allografts[8], and dog to rabbit non-vascularized xenografts[9]. The method has been applied clinically in the treatment of Kienbock's disease[10], talar avascular necrosis[3,11], scaphoid nonunion with avascular necrosis[12-14], and in prefabricated bone free flaps[15-17]. It also improves the results of necrotic structural allografts[18] and revascularizes massive structural allografts (intramedullary bone autograft)[19].

The potential of surgical angiogenesis to maintain viability of vascularized bone and joint allotransplants with and without short-term immunosuppression has been the focus of our previous research[20,21]. In one study, described in Chapter 2, we showed that short-term immunosuppression combined with a patent AV bundle is a prerequisite to retain viability[21]. In another study, described in Chapter 3, we demonstrated the long-term maintenance of viability in short-term immunosuppressed allotransplants[20]. In this study, we found isotransplants to behave as autotransplants, with measurable blood flow in the cortex, new vascular networks being formed radially from the implanted AV bundle, new bone formation, absence of signs of rejection, and normal osteocyte-filled lacunae.

The allograft group had minimal viable bone due to initial necrosis and incomplete creeping substitution at final follow-up. There was a strong inflammatory response due to the MHC mismatch, and bone remodeling was only seen adjacent to neoangiogenic blood vessels. These new vessels, forming from the AV bundle within the medullary canal did not reach the cortex, leading to no measurable cortical blood flow.

The vascularized allotransplants with a ligated AV bundle (Group 2) were expected to thrombose their nutrient vessels following withdrawal of Tacrolimus immunosuppression. The neoangiogenesis and resulting blood flow was small as a result, any new vessels the result of either AV bundle inosculation or ingrowth of new

vessels following its path. The hypovascularity is further reflected in the low bone viability score and absence of bone formation in this group.

Finally, the allotransplanted femora with patent AV bundles (Group 1) experienced enough time for capillary sprouting from the implanted AV bundles to traverse the medullary canal and penetrate cortical bone, reflected by measurable cortical blood flow, maintained despite withdrawal of immunosuppression and transplant pedicle rejection. The extent of capillary proliferation compared favorably to isotransplant controls. The vasculature provided a source of host-derived osteoblasts and osteocytes, maintaining bone turnover and bone viability (measurable bone formation; high bone viability grade, respectively). In the long-term, the only immunogenic tissue remaining is sequestered within the bone (leading to a low inflammation grade).

Some differences in blood flow, capillary density and histomorphometry were observed between groups, attributable to the independent variables by which they differ. Neoangiogenic capillaries reached the cortical edge more frequently in Group 1 than Group 4 bones. This resulted in higher cortical blood flow values, which are only detected near the cortical bone surface by the hydrogen sensing electrode. The overall capillary density was not different. As capillary density is measured within the entire bone volume, this demonstrates that the robust angiogenesis from Group 4 AV bundles had simply not advanced through the endosteal surface to the same extent as Group 1.

The femoral allotransplants (Groups 1 and 2) had greater immunogenicity than the allograft Group 3, where deep freezing resulted in values intermediate between allotransplants and isotransplanted femora (Group 4). Immune response within the bone is in turn dependent on access of circulating immune cells and antibodies via its circulation. Differences in both initial blood flow (present in the transplant Groups 1, 2 and 4, and absent in group 3), blood flow resulting from patent AV bundle angiogenesis and tissue immunogenicity would all be expected to modulate the rejection response.

Osteocyte viability is dependent upon either survival of transplanted allogeneic cells or their replacement resulting from later bone remodeling. The lineage of viable cells within long-term surviving bone allotransplants is of interest in transplanted

tissues. We have previously demonstrated graft chimerism to occur at high levels in rat femoral allotransplants using semi-quantitative PCR analysis[22]. A more accurate evaluation of osteocytes suggests that many of these cells are of recipient origin (results discussed in Chapter 8)[23]. Such chimeric replacement is made possible by the neoangiogenic capillary network, through which osteogenic cells migrate.

We used skin grafting as a means for evaluation of immune competence and to demonstrate the absence of donor-specific tolerance. The method is a good indicator of Class I mismatch. While flow cytometry and mixed lymphocyte reactions were not used, others have shown that medullary washout pre-transplant does not statistically reduce the Class II MHC load in experimental CTAs and as such our model is clinically relevant[24,25]. Skin has been shown to be the most immunogenic tissue in CTAs[26]. The results demonstrate the animals to be immunocompetent at 21 weeks, and without any donor-specific tolerance. Survival of the transplants is therefore explainable only by the development of a neoangiogenic circulation. If clinically applicable, it would greatly increase the safety of allotransplantation.

Methods to produce a state of tolerance recently have been at least partially successful, using systemic irradiation[27], monoclonal antibodies against adhesion molecules[28,29], or short-term FK-506 combined with 15-deoxyspergualin[30-32]. Tissue survival associated with donor specific tolerance has only recently been satisfactorily demonstrated, in rat hind limb and vascularized skin transplant models using monoclonal αβ T-cell receptor antibodies and a short course of either FK-506 or cyclosporine A[33-35]. Although mixed chimerism has been considered a promising method of tolerance induction, the method remains problematic for non-life critical transplants (see the case report and discussion in Chapter 12)[36]. The incidence of graft-versus-host disease (GVHD) after bone marrow transplantation has been reported to be 30-50% or more[37,38]. Transplantation of living musculoskeletal allotransplants containing bone marrow may carry some risk as well, although no evidence of GVHD has been found following hand transplantation[39]. From the practical point, the limitations and complications of tolerance are currently as onerous as those of immunosuppression. Neither is acceptable if widespread use of living musculoskeletal allotransplantation is to become practical or ethical.

We have described a novel method to maintain viability of bone allotransplants, without tolerance or long-term immunosuppression. In the present experimental study we have demonstrated that bone allotransplants may maintain bone blood flow without sustained immunosuppression or induction of tolerance by development of an autogenous neocirculation requiring only short-term FK-506. Ongoing research must focus in detail on the role of transplant chimerism, as well as evaluate mechanical properties after orthotopic transfer. Prior to clinical application additional obstacles to the use of CTAs must be overcome, including availability of donors and evaluating the risk of disease transmission. These practical considerations are similar to those in any organ transplant. Ultimately, we expect vascularized bone allotransplantation to become a viable and ethical method to reconstruct large bone defects.

Acknowledgment

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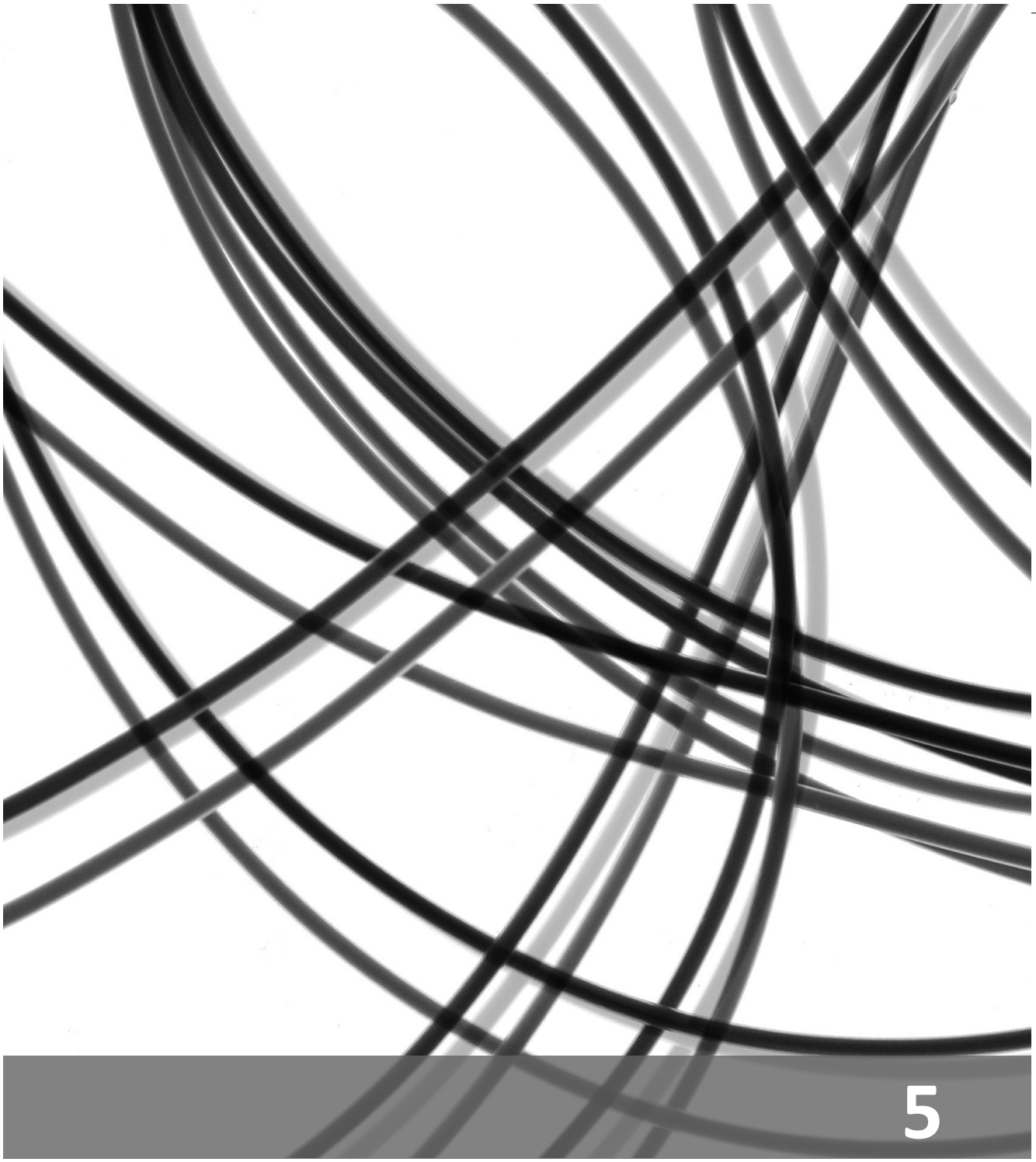

5

AUGMENTATION OF SURGICAL ANGIOGENESIS IN VASCULARIZED BONE ALLOTRANSPLANTS WITH HOST-DERIVED AV BUNDLE IMPLANTATION, BASIC FIBROBLAST GROWTH FACTOR AND VASCULAR ENDOTHELIAL GROWTH FACTOR ADMINISTRATION

Introduction

We have demonstrated the maintenance of femoral bone viability using a novel method of allotransplantation with replacement of the circulation through surgical angiogenesis (Chapters 2 and 3)[1,2], and have evaluated the underlying mechanisms by quantifying angiogenesis and blood flow as well as measuring graft chimerism[3]. We have shown that this method is independent of donor-specific tolerance induction, and is dependent on transplantation of a vascularized graft, with results comparable to isograft (Chapter 4).

The overall goal of the current study is to investigate growth factor enhancement of angiogenesis and new bone formation in our transplantation model. At surgery, we administered biodegradable microspheres encapsulating basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), or both within the transplanted femur adjacent to the AV bundle. We measured the effect of the growth factor(s) on angiogenesis, bone remodeling and bone blood flow.

Methods

Inbred female Dark Agouti (DA) rats (genetic expression: $RT1^a$, 150-175g) were used as donors in a nonsurvival procedure. Male Piebald Virol Glaxo (PVG) rats (genetic expression: $RT1^c$, 200-250g) were used as the recipient animals. This combination provided a strong histocompatibility mismatch. All animals were obtained from Harlan Sprague Dawley, Madison, WI. Immunosuppression was administered in the form of FK-506 (1mg/kg/day IM) (Tacrolimus, Fujisawa Pharmaceutical Co., Osaka, Japan).

Sterile technique was maintained throughout the procedures. All experiments were performed according to established National Institutes of Health guidelines and under the direction of our Institutional Animal Care and Use Committee. Animals were allowed to move freely in their cages and fed standard rodent feed and water *ad libitum*.

Animal Model

The group distribution is shown in Table 1. The transplantation procedure, bone blood flow and capillary density measurement procedures were identical to those described

in Chapter 2. Histologic grading of rejection and quantitative histomorphometry were performed as described in Chapter 4.

Growth factors were delivered as encapsulated poly(D,L-lactide-co-glycolide) (PLGA) microspheres placed on the AV bundle at the time of transplantation. The medullar canal was then filled with 20µl of collagen I solution at pH 7.4 and sealed with fibrin glue at each end (Tisseel VH Fibrin sealant, Baxter Healthcare, Westlake Village CA).

Table 1. Group Distributions

Group	Growth factor	N	Immuno-suppression	AV Bundle	Survival time
1	none	11	2 weeks	Patent	4 weeks
2	FGF2 10µg	10	2 weeks	Patent	4 weeks
3	VEGF 10µg	11	2 weeks	Patent	4 weeks
4	FGF2 10µg + VEGF 10µg	11	2 weeks	Patent	4 weeks

Microsphere manufacture

The fabrication process was done under aseptic conditions. Briefly, 1µl of a 1.0mg/ml growth factor solution (Trevigen Inc., Gaithersburg, MD), was emulsified in 2.5µl dichloromethane per mg PLGA (50:50 lactic to glycolic acid ratio and average molecular weight of 23kD) using a vortex at 3050rpm. Microspheres loaded with buffered saline were used for control groups. The mixture was re-emulsified in 1% w/v aqueous polyvinyl alcohol (PVA) solution to create a double emulsion. The content was then added to 0.3% w/v PVA and 2% w/v isopropanol solution and stirred for one hour. The extraction of the dichloromethane to the external alcoholic phase results in precipitation of the dissolved polymers and the formation of microspheres. The manufacturing parameters were previously determined by our collaborating laboratory[4,5] to provide a zero-order drug release for 28 days after an initial burst, on the condition of embedding in a substrate such as a collagen matrix. Each bone transplant was loaded with 15mg of microspheres containing 0.7µg per mg growth factor (=10µg total per transplant).

Assessment of microsphere surface morphology and size distribution

Microsphere morphology was assessed by scanning electron microscopy (SEM) (Hitachi S4700, San Jose, CA). For each group, the diameter of microspheres was

measured from three micrographs each containing 100-3000 microspheres using image analysis software (Scion Image for Windows 4.0.2; Scion Corporation, Frederick, MD), averaged and compared.

Data analysis

Continuous data, including capillary density, bone blood flow, and histomorphometry measures, were reported as means and standard deviations or medians and ranges, as appropriate. Categorical data, including histology, was described as medians and ranges. Capillary density, bone blood flow, and histomorphometry were compared across all four growth factor treatment groups, using analysis of variance or the Kruskal-Wallis test as appropriate. If a significant difference between groups was detected, the two sample t-test or Wilcoxon rank sum test was used to examine where the groups differed. Histology grade within the 4 groups was compared using the exact test for ordered contingency tables[6] and cumulative logistic regression[7]. All statistical tests were two-sided and the threshold of statistical significance was set at α=0.05. Analyses were performed with SAS, version 9.1 (SAS Institute Inc, Cary, NC).

Results

The mean (standard deviation) microsphere size per group was 73.7 (55.0)µm (Group 1, N=29), 66.2 (30.2)µm (Group 2, N=32), 56.8 (29.2)µm (Group 3, N=50) and 50.9 (32.3)µm (Group 4, N=34). There was no significant size variation (p=0.21, Kruskal-Wallis test). Overall morphology was similar for each microsphere type, with uniformly round contours and few perforations (Figure 1).

Further results are shown in Table 2. All of the saphenous AV bundles remained patent until sacrifice. There were no wound healing problems and all animals survived until the sacrifice date.

Bone blood flow varied significantly between groups (p=0.05, Kruskal-Wallis test) (Figure 2). Significantly more blood flow was found in Group 3 (VEGF) than Groups 1 (control) (p=0.031), 2 (FGF2) (p=0.05) and 4 (FGF2+VEGF) (p=0.001, Wilcoxon signed rank test). Group 2 also had more blood flow than Group 4 (p=0.001).

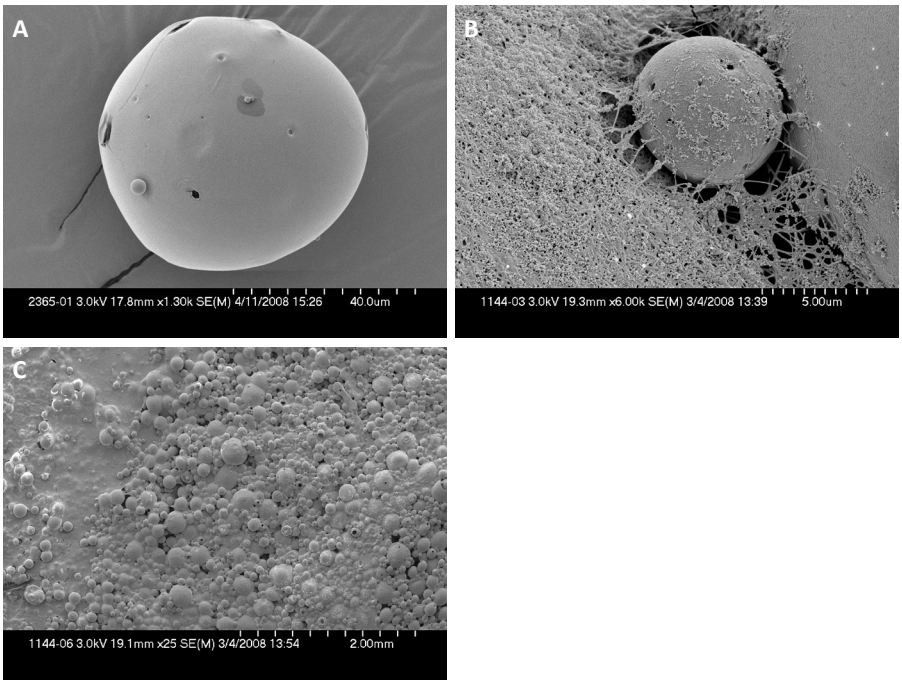

Figure 1. Scanning electron microscopy (SEM) of microspheres. (A) Single microsphere containing VEGF and FGF2 at 1300× magnification showing a smooth surface and uniform contour. (B) and (C) 6000× and 25× magnification, respectively, microspheres embedded in a polymerized collagen-I matrix.

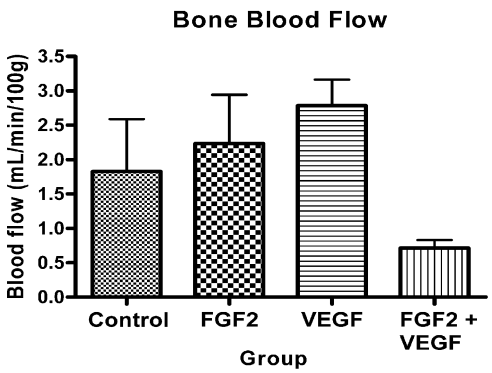

Figure 2. Bone blood flow as determined by hydrogen washout.

Table 2. Results. a: blood flow, ml/min/100g mean(range); b: capillary density, % mean(standard deviation); c: inflammation grade median(range); d: rejection grade or level of bone necrosis median(range); e: bone formation rate, $\mu m^3/\mu m^2$/year mean(range).

Group	Number	Blood Flow[a]	CapDens[b]	Inflam[c]	Rejec[d]	BFR[e]
1 – Control	11	1.8(0-5.4)	15.3(13.6)	3(2-4)	2(1-3)	23.5(0-105.2)
2 – FGF2	10	2.2(0-5.0)	24.3(17.1)	3.5(0-4)	2(1-3)	90.9(12.2-212.0)
3 – VEGF	11	2.8(0.7-4.8)	30.3(16.4)	3(1-4)	2(0-3)	96.8(16.4-154.9)
4 – FGF2+VEGF	11	0.7(0-1.3)	38.4(24.9)	4(1-4)	1(1-3)	93.2(0-211.7)

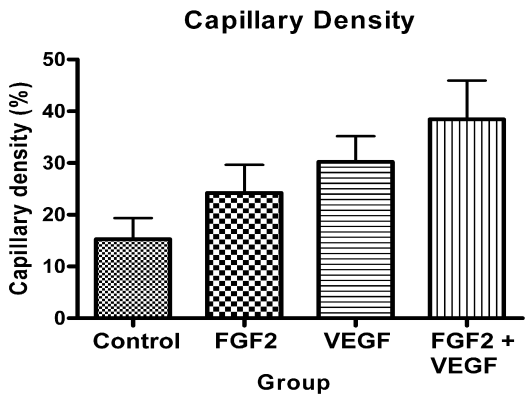

Figure 3. Capillary density as determined by microangiography.

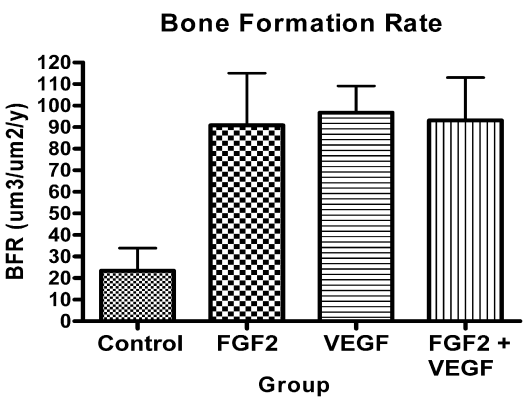

Figure 4. Bone formation rate as determined by quantitative histomorphometry.

Capillary density varied significantly between groups (p=0.039, analysis of variance). Group 4 showed significantly greater capillary density than Group 1 (p<0.05, Bonferroni multiple comparison test), and there was a significant linear trend (slope 3.78, R=0.43, p=0.0045) from Groups 1 to 4 (Figure 3).

Inflammation and bone viability did not vary significantly between groups (p<0.001, Cochran-Mantel-Haenszel test).

Bone formation varied significantly between groups (p=0.0056, Kruskal-Wallis test). Group 4 had more bone formation than Group 1 (p<0.001), Group 3 more than Groups 1 and 2 (p<0.05), and Group 2 more than Group 1 (p<0.01) (Wilcoxon signed rank tests) (Figure 4).

Discussion

Local administration of vascular growth factors significantly promotes angiogenesis; a steady-state release is more effective in doing so than a bolus injection[8,9]. One method by which continuous administration may be achieved is via PLGA microspheres[10]. Lower dosages are necessary to achieve a physiologic effect and microspheres protect the growth factor *in situ,* allowing interaction with target cells. They permit growth factors such as FGF2, whose half life is 50 minutes *in vivo*, to affect tissues that generally require 24 hours of exposure for response[11].

FGF2 is a powerful stimulator of neoangiogenesis *in vivo* and a pleiotropic regulator of vascular cell proliferation, migration and differentiation *in vitro*[12]. It promotes formation of larger and more complex blood vessels such as arterioles, and in bone simultaneously promotes osteoid formation[13]. Other angiogenic growth factors, such as VEGF, are specific only to endothelial cells. We have reported VEGF to promote angiogenesis in AV bundles implanted into necrotic bone, when delivered directly or with endothelial cell viral transfection[14,15]. FGF2 has also been used successfully in necrotic bone[12] and prefabricated hydroxyapetite molds[13]. VEGF works synergistically with FGF2 to stimulate angiogenesis *in vitro*[16] and *in vivo*[17]. Vascular remodeling in response to these growth factors is further dependant on arterial sufficiency and nitric oxide production[18]. We found significantly improved cortical blood flow with the application of VEGF over control, and over FGF2. FGF2 provided increased blood flow, although this was not significant. We did not find the expected synergistic effect

of FGF2 and VEGF on blood flow. The apparent disconnect between greater capillary density and lower blood flow seen when FGF2 and VEGF are combined is of interest. We speculate that the large number of capillaries formed in the combined group may have resulted in relatively lower regional flow rates, when a constant total bone blood flow is apportioned between more vessels. Alternatively, capillary formation ending blindly without venous outflow could also result in poor measured regional flow. Capillary density measurements in fact measure the total vascular volume within both cortical and medullary bone, while hydrogen washout measurements are limited to superficial cortical bone. The capillary density was significantly greater in the combined growth factor group, which provides evidence that the two exert a synergistic effect *in vivo.*

Histologic grading of rejection was similar across all groups, as were levels of bone viability by osteocyte counts. This serves as further control for any deleterious effects of growth factor administration. Bone viability and capillary density did not vary significantly in the control group from previous groups with vascularized allografts and AV bundle implantation, in which no microspheres were used (Chapter 3) (N=11)[1]. This serves as a control for any measurable effect of the presence of microspheres in the medullar canal, or of the waste products after their hydrolysis (a pH decrease from lactic and glycolic acid may have affected AV bundle patency or the rate of neoangiogenesis and bone formation). Blood flow and viability was uniformly greater than in non-immunosuppressed grafts from previous studies using this experimental model (Chapters 2 and 3) (N=23[2]; N=11[1]).

FGF2 provided more bone formation than control and VEGF more than both control and FGF2 groups. Both factors combined gave similar results to the VEGF group. These results imply that new bone formation is a factor dependent on angiogenesis. If we could manufacture microspheres that sequentially released the two growth factors, or mix microspheres manufactured with different formulations to provide different release characterisitcs[19], we may better mimic the in vivo situation where FGF2 is expressed later in bone formation that VEGF[20].

Clinical use of vascularized allografts requires a method that maintains long-term tissue viability, including measurable blood flow, osteocyte viability, active bone remodeling and healing response, and maintained biomechanical properties. Safety in such non-life-critical tissues is paramount. Current immune modulation methods carry

significant risks, including graft-versus-host (GVH) disease, opportunistic infection and carcinogenesis. Most published research makes use of immunosuppressive drugs and/or efforts to induce a tolerant state. The possibility of eliminating such long-term immune modulation by development of a neoangiogenic host circulation within the transplanted bone is the focus of this research. Here we demonstrate the feasibility of this technique with the enhancement of neoangiogenesis and new bone formation through the local delivery of angiogenic cytokines.

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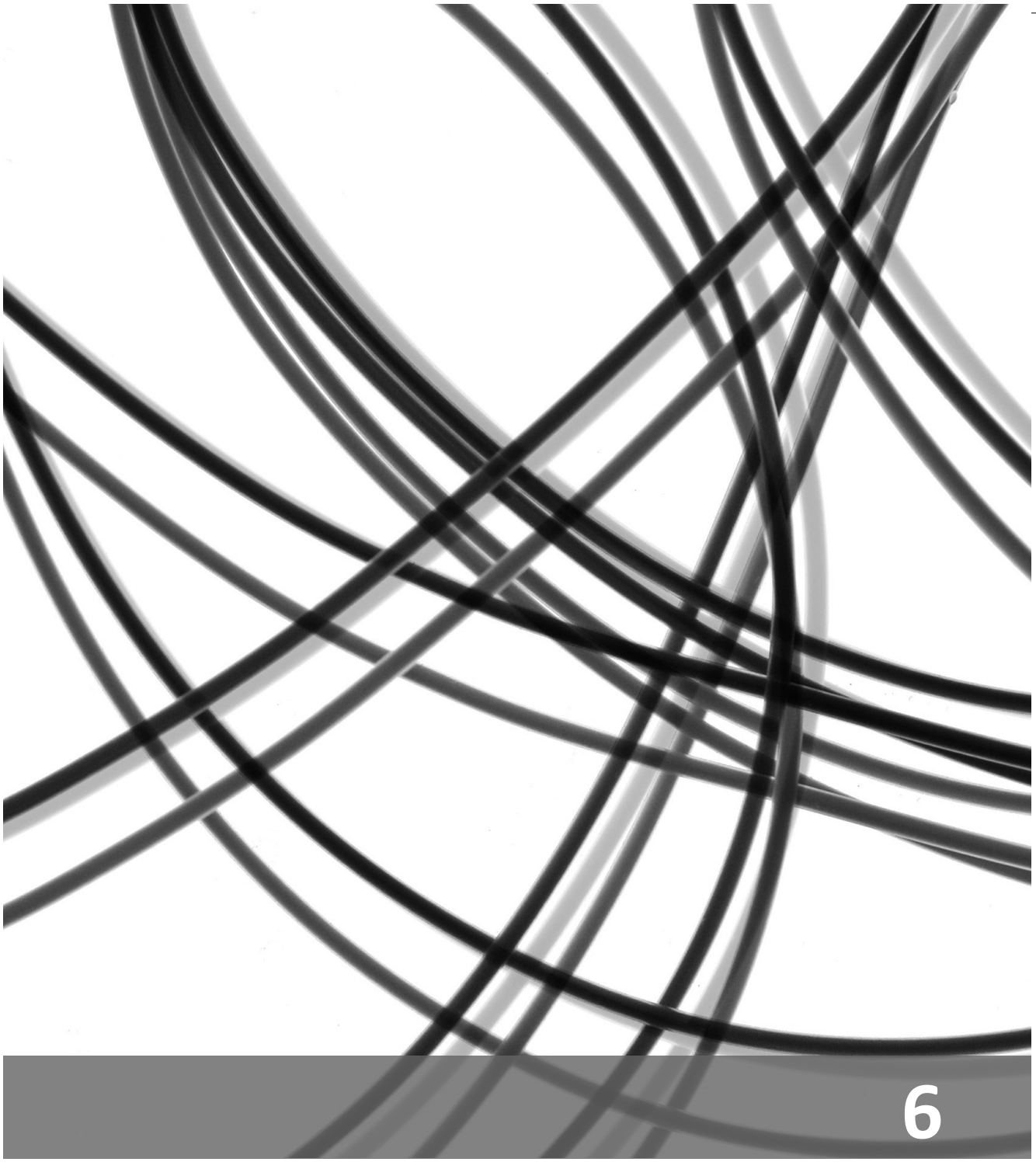

6

LONG-TERM EFFECTS OF FIBROBLAST GROWTH FACTOR-2 AND VASCULAR ENDOTHELIAL GROWTH FACTOR ADMINISTRATION ON ANGIOGENESIS AND BONE FORMATION IN VASCULARIZED BONE ALLOTRANSPLANTS

Introduction

We have investigated a novel method to enable transplantation of living bone without long-term immune modulation (see Chapters 2 – 4). This is accomplished by placing vascularized recipient tissue in the form of an arteriovenous (AV) bundle or fascial flap within the bone at the time of transplantation, together with microvascular repair of nutrient vessels. Only short-term immune modulation is necessary while neoangiogenesis from host tissue occurs. We have enhanced angiogenesis and new bone formation through the administration of biodegradable microspheres encapsulating basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), or both within the transplanted femur adjacent to the AV bundle (Chapter 5). Short-term (4 weeks) survival analysis showed augmented angiogenesis, bone formation and bone blood flow compared to controls containing microspheres loaded with buffer solution[4].

In the current study, we employed the same model, and measured the long term (18 weeks) effects of FGF2, VEGF and a combination on angiogenesis, bone remodeling and bone blood flow in vascularized bone allotransplants.

Methods

Inbred female Dark Agouti (DA) rats (genetic expression: $RT1^a$, 150-175g) were used as femoral allotransplant donors in a nonsurvival procedure. Male Piebald Virol Glaxo (PVG) rats (genetic expression: $RT1^c$, 200-250g) were used as the recipient animals. This combination provided a strong histocompatibility mismatch. All animals were obtained from Harlan Sprague Dawley, Madison, WI. Immunosuppression was administered in the form of FK-506 (1mg/kg/day IM) (Tacrolimus, Fujisawa Pharmaceutical Co., Osaka, Japan). The independent variable evaluated was growth factor administration, resulting in four experimental groups (none, FGF2, VEGF or FGF2 + VEGF, see also Table 1).

Sterile technique was maintained throughout the procedures. All experiments were performed according to established National Institutes of Health guidelines and under the direction of our Institutional Animal Care and Use Committee. Animals were allowed to move freely in their cages and fed standard rodent feed and water *ad libitum*.

The animal model, operative procedure, microsphere manufacture, blood flow measurement, microangiography and histologic grading of rejection were identical to that described in Chapter 5.

Table 1. Group Distributions

Group	Growth factor	N	Immuno-suppression	AV Bundle	Survival time
1	none	11	2 weeks	Patent	18 weeks
2	FGF2 10μg	10	2 weeks	Patent	18 weeks
3	VEGF 10μg	11	2 weeks	Patent	18 weeks
4	FGF2 10μg + VEGF 10μg	11	2 weeks	Patent	18 weeks

Quantitative histomorphometry for bone remodeling

Calcein and tetracycline hydroxychloride were given by perivascular injection near the tail vein (both 20mg/kg), 2 weeks and 2 days prior to sacrifice, respectively. A semiautomatic image analysis system (Osteomeasure®; Osteometrics, Atlanta, GA) was used for quantitative histomorphometric assessment of new bone formation and resorption[12]. Perimeters of trabecular bone surface, osteoblast-covered surface, eroded surface, and osteoclast-covered surface were measured at the endosteal level and periosteal level at 200× optical magnification. Bone formation rates were calculated for each level separately. This is the essential difference from the method described in Chapter 4.

Alkaline phosphatase activity

The alkaline phosphatase activity was measured using a kit (Sigma Chemical Co.). Bone samples were rinsed with PBS, harvested, and processed for determining the alkaline phosphatase activity. The activity was normalized to total cellular protein, which was determined by the Bradford protein assay.

Data analysis

Continuous data, including capillary density, bone blood flow, and histomorphometry measures, were reported as means and standard deviations or medians and ranges, as appropriate. Categorical data, including histology, was described as medians and ranges. Capillary density, bone blood flow, and histomorphometry were compared

across all four growth factor treatment groups, using analysis of variance or the Kruskal-Wallis test as appropriate. If a significant difference between groups was detected, the two sample t-test or Wilcoxon rank sum test was used to examine where the groups differed. Histology grade within the 4 groups was compared using the exact test for ordered contingency tables[13] and cumulative logistic regression[14]. All statistical tests were two-sided and the threshold of statistical significance was set at α=0.05. Analyses were performed with SAS, version 9.1 (SAS Institute Inc, Cary, NC).

Results

Results are shown in Table 2. All of the saphenous AV bundles remained patent until sacrifice. There were no wound healing problems and all animals survived until the sacrifice date.

Table 2. Results. a: blood flow, ml/min/100g mean(range); b: capillary density, % mean(standard deviation); c: inflammation grade median(range); d: viability grade expressed as level of bone necrosis median(range); e: bone formation rate, $\mu m^3/\mu m^2$/year mean(range); f: Alkaline Phosphatase activity, units mean(range).

Group	Number	Blood Flow[a]	CapDens[b]	Inflam[c]	Viability[d]	BFR[e]	Alk Fos[f]
1 – Control	11	0.22 (0-1.09)	19.5 (15.9)	0(0-2)	2(1-3)	0(0-0)	1135 (238-2121)
2 – FGF2	10	0.41 (0-1.09)	30.2 (18.0)	1(0-3)	1(1-3)	22.2 (0-158.9)	1553 (30-6583)
3 – VEGF	11	0.48 (0.0-1.53)	10.6 (11.4)	2(0-3)	1.5(1-3)	1.8 (0-7.5)	893 (101-2826)
4 – FGF2 + VEGF	11	1.16 (0-3.92)	12.2 (8.1)	2(1-3)	2(1-3)	4.9 (0-49.2)	791 (148-3097)

Bone blood flow varied significantly between groups (p=0.026, Kruskal-Wallis test) (Figure 1). Significantly more blood flow was found in Group 4 (FGF2+VEGF) than Groups 1 (control) (p=0.004), and 3 (VEGF) (p=0.04, Wilcoxon signed rank test). Group 4 also had more blood flow than Group 2 (FGF2), although this was not significant (p=0.131). No further significant differences between groups were found.

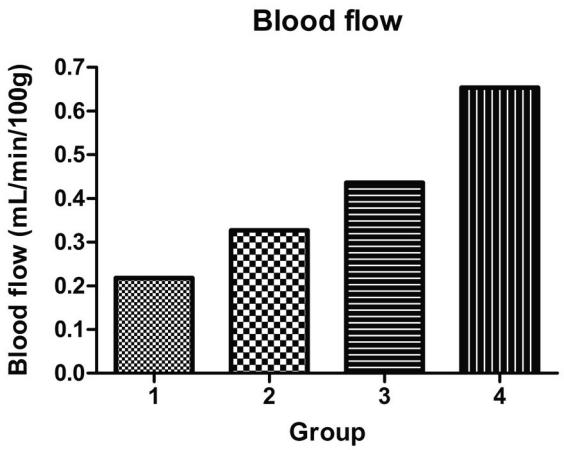

Figure 1. Bone blood flow as determined by hydrogen washout.

Capillary density varied significantly between groups (p=0.017, analysis of variance) (Figure 2).

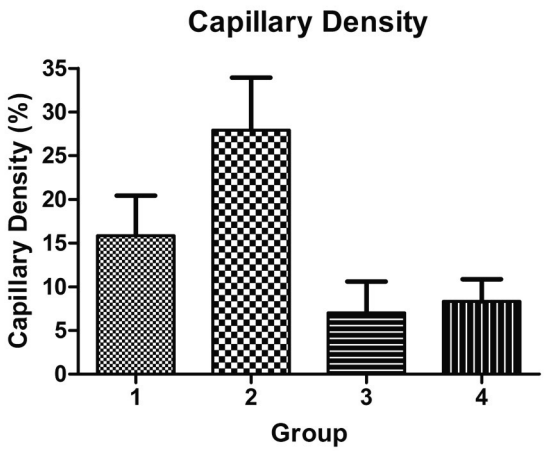

Figure 2. Capillary density as determined by microangiography.

Group 2 showed significantly greater capillary density than Groups 3 and 4 ($p<0.05$, Bonferroni multiple comparison test). Group 2 also had greater capillary density than Group 1, although this was not significant ($p>0.05$).

Bone viability did not vary significantly between groups ($p<0.001$, Cochran-Mantel-Haenszel test). Inflammation grade was significantly lower in Group 1 than Groups 3 and 4 ($p<0.05$) (Figure 3).

Neither bone formation in both the endosteal plane and periosteal plane, nor alkaline phosphatase activity varied significantly between groups ($p>0.05$, Kruskal-Wallis test).

The only positive and significant correlation between measures of circulation and bone formation was between the alkaline phosphatase activity and bone blood flow values in Group 3 ($R^2=0.83$, $p<0.05$).

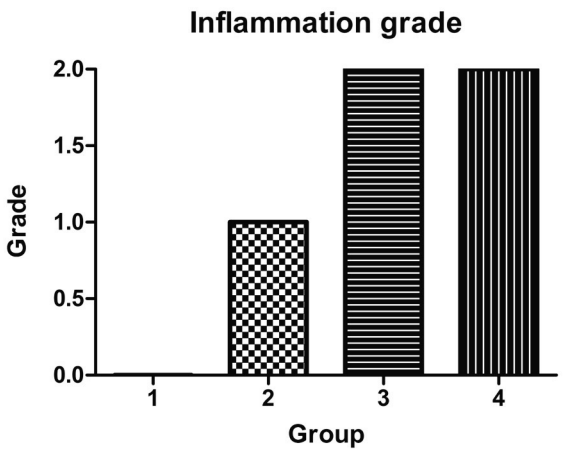

Figure 3. Inflammation grade.

Discussion

We previously found significantly improved cortical blood flow with the application of VEGF over control, and over FGF2 (Chapter 5)[4]. In that 4-week survival study, we did not find the expected synergistic effect of FGF2 and VEGF on blood flow. In the current long-term survival study, this synergistic effect was finally revealed. Also previously apparent was a disconnect between greater capillary density and lower blood flow

seen when FGF2 and VEGF were combined. We speculated that the large number of capillaries formed in the combined group may have resulted in relatively lower regional flow rates, when a constant total bone blood flow is apportioned between more vessels. After 18 weeks, however, the opposite is seen: FGF2+VEGF resulted in significantly lower capillary density than FGF2 alone, and similar to VEGF alone, while blood flow values were higher. Thus the discord between capillary density and blood flow remains, but is reversed at some point between 4 and 18 weeks. Presumably the large immature capillary network at 4 weeks is replaced by a more stable system capable of transporting greater volumes of blood per weight of tissue at 18 weeks.

Also in contrast to the short-term results is the finding of significantly higher levels of inflammation in Groups 3 and 4. Whether this is another cause or an effect of the greater blood flow values is unclear. The byproducts of microsphere degradation being the same in amount and type in all groups (lactic and glycolic acid), this difference cannot be explained as a reaction to increased acidity after microsphere breakdown.

On long-term follow-up, we found no evidence of growth-factor-induced bone formation. In Chapter 5 we had demonstrated that FGF2 provided more bone formation over control and VEGF more than both control and FGF2 groups[4]. In order to verify the lack of increased bone formation after 18 weeks, it was assessed in both the endosteal plane and periosteal plane, and further evidence searched for using a biological marker of bone deposition, alkaline phosphatase. None of these values were able to prove a relationship between bone formation and growth factor administration, however.

In conclusion, administration of vasculogenic cytokines in vascularized allotransplants via biodegradable microsphere encapsulation positively influences cortical bone blood flow in the long term. Capillary density values diminish as cortical blood flow increases, possibly reflecting maturation of the vascular bed. Further studies are needed in order to determine the relationship between cortical blood flow and capillary density as a function of time.

New bone formation did not increase in the wake of vascular stimulation with FGF2 and/or VEGF at 18 weeks. A combination of local sustained BMP-2 release from microspheres and VEGF from a hydrogel has been shown previously to significantly enhance ectopic bone formation compared to BMP-2 alone[15]. Similar studies using

microspheres loaded with a combination of vasculogenic and bone morphogenic proteins may provide combined vessel and bone stimulation with augmented bone formation rates over control in our model in the long term.

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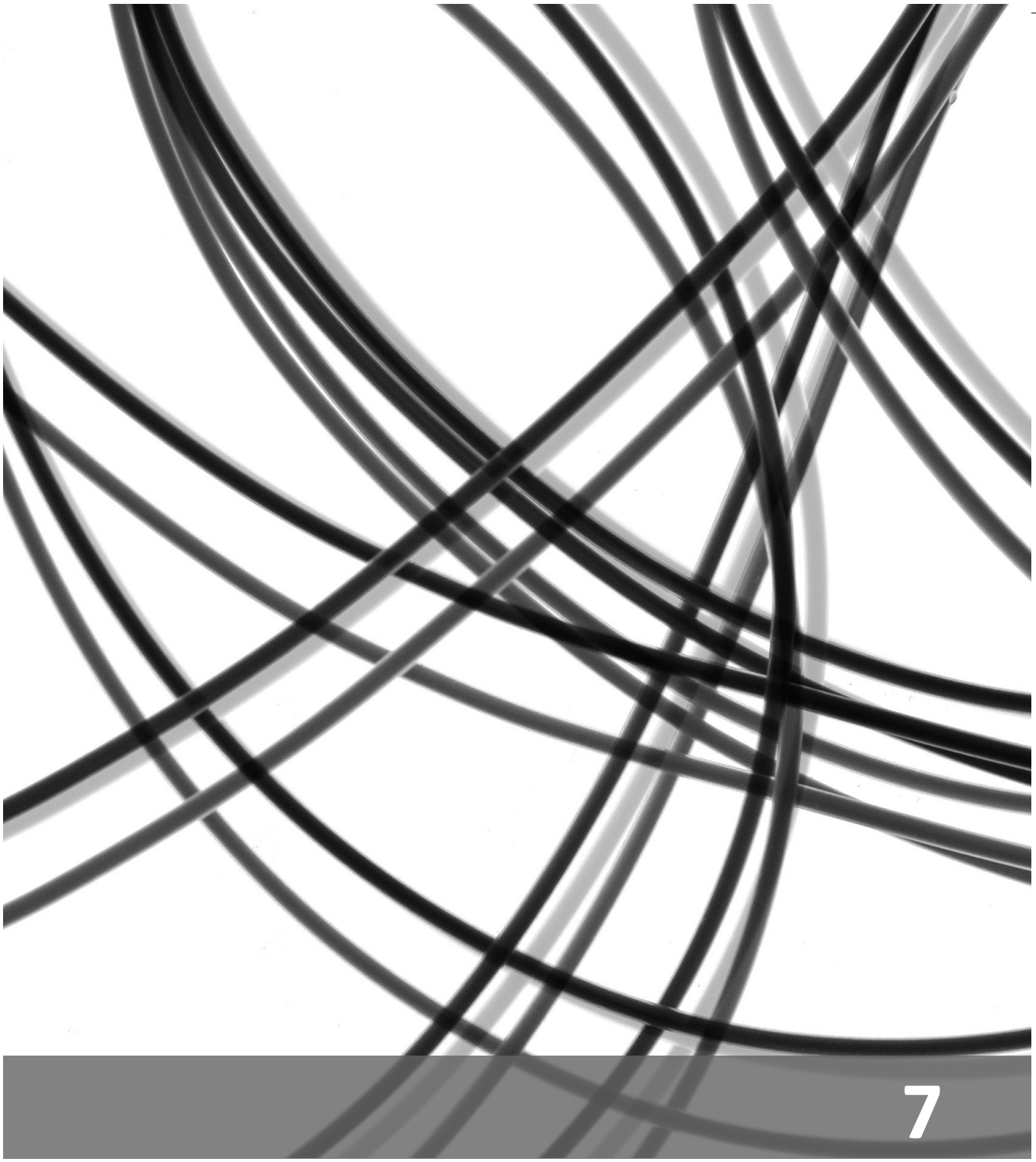

7

REVASCULARIZATION AND BONE REMODELING OF FROZEN ALLOGRAFTS STIMULATED BY INTRAMEDULLARY SUSTAINED DELIVERY OF BASIC FIBROBLAST GROWTH FACTOR AND VASCULAR ENDOTHELIAL GROWTH FACTOR

Introduction

Conventional (nonvascularized) bone allografts are frequently used for large bone reconstructions[1]. Freezing, freeze drying, irradiation, autoclaving and treatment with various chemical reagents can be used to reduce disease transmission and diminish immunogenicity of the allograft[2]. Deep freezing remains the most frequently used method to reduce recipient antigenic reaction and induce incorporation of the graft[3]. The process largely devitalizes bone due to cellular membrane damage by crystallization. Such a necrotic graft thus serves primarily as structural support readily allowing for weight bearing[4]. The bone maintains its osteoconductive properties, but the freezing reduces osteoinductive potential, defined as the intrinsic capacity to stimulate new bone formation. In addition, revascularization of the frozen graft is minimal, with resultant insufficient remodeling leading to weakening of the graft over time[3,5]. In such necrotic grafts, remodeling is entirely dependent on creeping substitution: a slow and incomplete repopulation of the necrotic graft by vessels and viable osteocytes of the recipient. Fractures can consequently occur even in well incorporated grafts many years after transplantation due to incomplete remodeling[6,7]. Other frequent complications include infection and non-union[8], which require revision surgery or amputation.

It is possible that accelerated and improved revascularization of the graft may improve outcomes after conventional structural bone allografting. This may be accomplished by implanting or wrapping the necrotic bone with well-vascularized tissue, possibly combined with the local, continuous delivery of angiogenic or osteogenic growth factors. The resultant angiogenesis, defined as the formation of new capillaries from an existing circulation, invades necrotic trabeculae. This both accelerates and extends the remodeling process. In this study, the desired cytokine is delivered by release from biodegradable poly (D,L-lactide-co-glycolide) (PLGA) microspheres[9], combined with revascularization from an implanted saphenous AV bundle to facilitate angiogenesis.

As in Chapter 6, for this study VEGF and FGF2 were used, the former for its strong angiogenic potential and contribution to osteoblast proliferation[10,11,12], and the latter for its roles in osteoblast differentiation, bone formation and angiogenesis[13,14]. As previously mentioned, their combined use may have a synergistic effect on angiogenesis in vivo[15]. Here we examine the single and combined effect of these growth factors on conventional graft revascularization and osteogenesis. This is an

essential and clinically feasible intermediate step towards eventual application of our allotransplantation model to human subjects.

Materials and Methods

Growth Factor Preparation

Four groups of 10 rats per group were used: Group 1 served as control and received microspheres with phosphate buffered saline (PBS). Groups 2 and 3 received FGF2 and VEGF, respectively, and Group 4 received FGF2 and VEGF combined.

Bone Transplantation Procedure

The same animal model as described in Chapters 5 and 6 was used. The only difference was the bilateral harvest of non-vascularised grafts from the female Dark Agouti rat donors ($RT1^a$) and the absence of microsurgical anastomoses in the recipient rats (Figure 1).

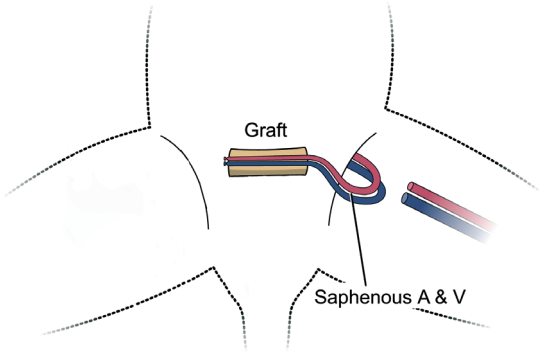

Figure 1. Heterotopic transplantation model. The graft is transplanted to a subcutaneous abdominal pocket with the saphenous AV bundle inserted into the medullary canal. The microspheres are inserted alongside the AV bundle.

Grafts were reamed with a 2 mm hand drill, stripped of excess tissue, rinsed with saline and stored without cyroprotectants at -80 ^{O}C. Prior to transplantation to the male Piebald Virol Glaxo rats ($RT1^c$), the grafts were thawed in sterile saline at room temperature. As described in Chapters 5 and 6, they were then wrapped in a reinforced silicone membrane, the left saphenous arteriovenous (AV) bundle was inserted into

the medullary canal and microspheres mixed with Collagen I were injected alongside the AV bundle. Grafts were placed heterotopically into an abdominal subcutaneous pocket. During 14 days postoperatively, FK-506 (Fujisawa Pharmaceutical Co., Osaka, Japan) immunosuppression was administered at a dose of 1mg/kg/day IM. All animals were treated according to directions of the Institutional Animal Care and Use Committee.

Bone blood flow, microangiography and quantitative histomorphometry analysis were performed as described in Chapter 5.

Osteocyte repopulation

Haematoxylin & Eosin stained transverse whole sections were examined at 400× magnification. The number of osteocyte-filled lacunae compared to the total number of lacunae was counted for 10 random fields in each section and presented as a percentage, corresponding to osteocyte repopulation. A lacuna was considered filled if it was occupied with an osteocyte with a normal nucleus and normal cytoplasmic staining.

Statistics

Results were analyzed using the Kruskal Wallis test and the Cochran-Mantel-Haenszel test as appropriate. If a significant difference between groups was detected, the Wilcoxon rank sum test was used to investigate the difference between the individual groups. Correlation between dependent variables was assessed with Spearman's rank correlation coefficient. Significance was set at $\alpha = 0.05$. Statistical analyses were performed with SAS, version 9.1 (SAS Institute Inc, Cary, NC).

Results

No wound healing problems were encountered and all animals survived until the sacrifice date. At sacrifice 85% of the animals had a patent AV bundle: 1 animal in Group 1, 2 in Group 3 and 3 in Group 4 had a thrombosed AV bundle and were excluded from further analysis. The individual group results are summarized in Table 1. Cortical bone blood flow was significantly higher in rats treated with VEGF and FGF2+VEGF compared to control. Although Group 2 (FGF2) had higher bone blood flow than control, this difference was not significant.

Neoangiogenesis was observed in all groups. Although capillary density was higher in all growth factor treated groups, there were no significant differences as compared to control.

At 4 weeks, osteocyte repopulation as measured from H&E sections was modest with an overall range of 0% to 11%. Groups 1, 2, 3 and 4 had a mean of 2.56%, 3.40%, 4.50% and 4.29% viable cells populating the lacunae, respectively. No significant differences were detected between the groups.

Quantitative histmorphometry demonstrated bone turnover parameters that were higher in the three groups that received growth factors as compared to control. However, only Group 3 (VEGF) and 4 (FGF2 + VEGF) had significantly higher mineral apposition rates (MAR) and bone formation rates per bone surface area (BFRBS) as compared to Group 1 ($p < 0.05$). Group 3 additionally had a significantly higher mineralizing surface per bone surface (MS/BS). Combining all experimental groups, the correlation coefficient between mineral apposition rate and bone blood flow (R) was 0.49 ($p = 0.009$).

Table 1. Individual group results, expressed as means +/- standard deviation. Outcomes that differed significantly from control (Group 1) are underlined.

Group	1	2	3	4
Growth Factor	None (PBS)	FGF-2	VEGF	FGF-2+VEGF
Bone Blood Flow (ml/min/100gr)	0.39 +/- 0.36	2.39 +/- 1.78	3.84 +/- 2.01	3.85 +/- 2.22
Capillary Density (%)	7.39 +/- 6.25	10.84 +/- 3.59	9.76 +/- 6.85	8.48 +/- 6.37
MS/BS (%)	1.63 +/- 1.06	2.70 +/- 1.8	3.39 +/- 1.14	2.99 +/- 1.46
MAR (um/day)	0.45 +/- 0.14	0.59 +/- 0.12	0.67 +/- 0.11	0.66 +/- 0.12
BFRBS ($\mu m^3/\mu m^2$ per year)	3.48 +/- 2.11	6.51 +/- 2.98	8.18 +/- 2.62	8.27 +/- 3.32
Histologic score (%)	2.56 +/- 2.50	3.40 +/- 2.59	4.50 +/- 2.45	4.29 +/- 3.04

Discussion

Revascularization of conventional allografts is required to allow adequate bone remodeling and achieve active bone formation throughout the complete graft[16]. We present a model with a surgically revascularized bone allograft that is continuously exposed to growth factors released from biodegradable microspheres. Various other methods of experimental growth factor delivery have been tested, yet most lack the advantages that are offered by the delivery of biodegradable microspheres. Because of the short half life of FGF2 and VEGF in vivo[17,18], and the need for prolonged exposure to sustain a minimal bioactive response, slow and uninterrupted release of growth factors from microspheres is desirable, and gives potential for future clinical application[18,19]. PLGA microspheres furthermore can be delivered to the exact target site and provide a highly localized growth factor release without adverse systemic effects[18].

We found that VEGF increased cortical bone blood flow as well as bone turnover, even in this short-term survival study, confirming its potential to augment conventional allograft healing. VEGF has already proven to be a useful cytokine in fracture, non-union and distraction models in previous animal studies[20,10,21]. However, few studies have been performed with continuous and local exposure of VEGF to a nonviable allograft. Suzuki described the effect of a 3-day continuous VEGF infusion in a conventional allograft model with an implanted AV bundle, finding an angiogenic response in the first week only and no effect thereafter[22]. In a gene transfection mouse model, Ito only found significantly increased revascularization and bone remodeling when VEGF was combined with receptor activator of nuclear factor kB ligand (RANKL)[23]. Previous work from our laboratory involved VEGF gene transfer to conventional allografts with implanted AV bundles and found increased bone blood flow only initially at 1 week; no difference was discovered al later time points[24]. The increase in bone remodeling and enhanced angiogenesis after sustained VEGF exposure found in this study is a promising finding for future therapeutic approaches in conventional graft revitalization.

FGF2 has proven to be a variably effective osteogenic cytokine in frozen allografts[25,26,27]. Lamerigts et al described an angiogenic effect of FGF2 in frozen bone allografts that we were unable to reproduce. The optimal *in vivo* concentration of FGF2 is unknown.

The range of effective doses of FGF2 may be narrower than other growth factors, with high doses actually reducing osteoblastic activity[28,29,30,25]. Although microspheres do release growth factors in a continuous and controlled fashion, the initial burst release that occurs with this method in the first 24 hours might adversely affect osteoblast development[31,11,29]. Other doses or methods of delivery than used in this study might elicit a more robust angiogenic or osteogenic response. FGF2 may also cause a persistent proliferative state in osteoblastic development without differentiation of osteoprogenitor cells[26]. We placed the frozen femoral allograft heterotopically in an abdominal pocket, as this allows isolation of the bone in a silicone sheet to better observe the intrinsic effect of the AV bundle angiogenesis. Orthotopic placement would allow weight bearing and contact with viable recipient bone marrow, better simulating clinical conditions. FGF2 could hypothetically be more effective in such an environment, with abundant nutrients and osteoprogenitor cells present[32].

Previously, the synergistic effect of FGF2 and VEGF on angiogenesis has been described in ischemic hindlimb animal models[15,33]. As in Chapter 5, we did not find such synergism in this study. Instead, the combination of growth factors showed equal angiogenic and osteogenic potential to VEGF alone. While dose, delivery method and time of administration are all potentially important variables, within our parameters there was no angiogenic and osteogenic benefit to combined administration at 4 weeks. If the survival time were extended to 18 weeks, as in Chapter 6, the synergistic effect may have become more apparent.

Osteocyte repopulation suggested improved bone remodeling with higher numbers of viable osteocytes in all growth factor groups, but the differences did not prove to be significant. Four weeks is a short time for structural bone remodeling, and larger differences would reasonably be expected at longer survival periods[34]. The correlation found between bone blood flow and mineral apposition rate indicates the influence of revascularization on the bone remodeling process.

In conclusion, VEGF proves to be a potent growth factor as delivered by biodegradable microspheres. It stimulates angiogenesis and osteogenesis in otherwise necrotic bone grafts. VEGF encapsulated in PLGA microspheres therefore offers a promising future therapeutic approach to enhance revascularization and bone remodeling in large segmental allografts. In this study no synergistic effect was found when combining VEGF with FGF2. Future research should focus on optimal concentrations and combinations of growth factors necessary to enhance conventional graft incorporation.

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8

REPOPULATION OF VASCULARIZED BONE ALLO-TRANSPLANTS WITH RECIPIENT-DERIVED CELLS: DETECTION BY LASER CAPTURE MICRODISSECTION AND REAL-TIME PCR

Introduction

Allotransplantation of living musculoskeletal tissue remains experimental, due to unacceptable risks of long-term immune modulation currently required to maintain tissue viability[1]. A possible solution to this problem could be to develop a neoangiogenic circulation within a transplanted bone by implantation of recipient-derived vessels. As previously discussed, we have demonstrated the ability of this method to maintain bone blood flow and osteocyte viability[2]. Investigation of the mechanisms underlying these observations requires study of transplant chimerism, defined as the movement of cells from the recipient into the transplanted bone. For this purpose, we determined the relative proportion of the Sry gene (recipient-derived) to an autosomal housekeeper gene (cyclophilin) in our sex-mismatched transplantation model. We hypothesize that: 1) investigation of transplant chimerism is possible in tissue allotransplantation with our methods, 2) this process is moderated by short term immunosuppression and promoted by patent AV bundle implantation and 3) transplant chimerism correlates with bone blood flow and bone viability.

Methods

Vascularized femoral allotransplantations were performed from female Dark Agouti (DA: genetic expression: $RT1^a$) rats to male Piebald Virol Glaxo (PVG: genetic expression: $RT1^c$) rats, representing a major immunohistocompatability mismatch. Sex-mismatched transplantation was performed to aid in later identification of cellular lineage within the transplanted tissue. An 18 week survival period was chosen, based upon our previous work, which demonstrated substantial repopulation of transplanted rat femora at this time period[3]. All experiments were performed under the direction of our Institutional Animal Care and Use Committee.

Surgical Procedure

The surgical procedure has been described previously (see Chapter 2)[2]. At surgery, each recipient rat was randomly allocated to one of 4 groups, which differed in the use of immunosuppression (none versus short-term Tacrolimus) and patency of implanted recipient vessels for neoangiogenesis (patent versus ligated saphenous AV bundles) (Table 1).

Table 1. Demographics. Group 1 (patent AV bundle, no immunosuppression (IS); Group 2 (ligated AV bundle, no IS); Group 3 (patent, Tacrolimus IS); Group 4 (ligated AV bundle, Tacrolimus IS). *Specimens with enough tissue left for DNA extraction.

Group	AV-Bundle	Immunosuppression	n	N left for relative expression ratio measurement*
1	Patent	None	11	10
2	Ligated	None	7	4
3	Patent	TACROLIMUS, 2 weeks	11	10
4	Ligated	TACROLIMUS, 2 weeks	9	7

Bone Blood Flow

At 18 weeks the hydrogen washout method, as described in Chapter 10[4] was used to determine bone blood flow.

Histology

After euthanization, the transplant was carefully removed. A transverse section was decalcified and stained with hematoxylin/eosin. Bone viability was measured using a grading system based upon osteocyte counts (the percentage of lacunae either vacant or occupied by an osteocyte) on the complete transverse histologic cross-sections (200× magnification). Two samples were graded from each animal and the average taken. A four-point scale was used, ranging from a score of 0 (no osteocyte necrosis) to 3 (all lacunae empty), as described in Chapter 3[2].

Laser Capture Microdissection

Decalcified, formalin-fixed and paraffin-embedded 8 µm sections were placed on metal-framed polyethylene naphthalate (PEN) membrane slides (Arcturus Bioscience, Inc., Mountain View, CA). After deparaffinization for 2 minutes in xylene and air drying, the membrane slide was placed in the Veritas Laser Capture Microdissection System (Model #704, Molecular Devices, Inc., Sunnyvale, CA). Using the UV laser cutting feature, a region of cortical bone averaging 1.21 μm^2 was taken from the samples (Figure 1). The cortical bone samples were captured on a specialized cap (CapSure Macro LCM caps, Arcturus Bioscience, Inc., Mountain View, CA) and were then ready for DNA extraction. Using stable Proteinase K as an extraction agent, (PicoPure DNA Extraction Kit, Arcturus Bioscience, Inc., Mountain View, CA) and 16 hours of incubation at 65°C, DNA was recovered from the sample.

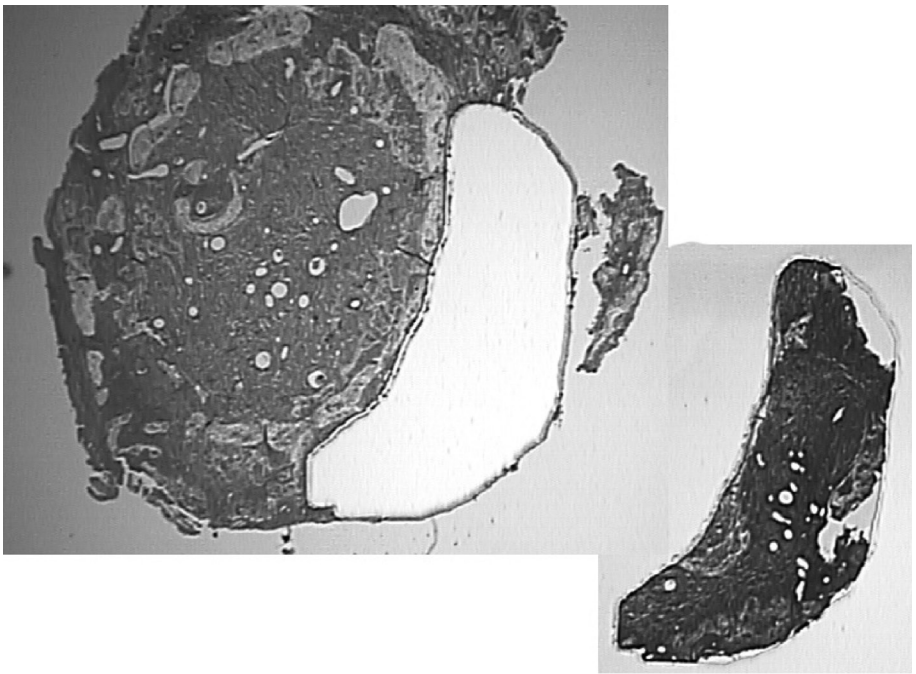

Figure 1. Specimen after microdissection showing the captured section used for real-time qPCR.

Spin columns (Performa spin columns - Catalog # 13266, Edge Bio Systems, Gaithersburg, MD) were used to further purify the extracted product, which averaged 13.9 ng/µl DNA. This procedure involved preparing a Performa gel filtration cartridge by centrifuging at 750×G for 2 minutes and then transferring the cartridge to a 1.5 ml microcentrifuge tube. Afterwards, the sample was added drop-wise to the center of the packed column and centrifuged again for 2 minutes at 750×G. The eluate was retained and frozen in a -20° C freezer for further evaluation.

Quantitative real time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (real-time qPCR) was performed using a Bio-Rad MyiQ Real-Time Instrument and Bio-Rad Sybr Green Super mix (Bio-Rad Laboratories catalog # 170-8880, Hercules, CA.). Genomic DNA was extracted from paraffin slide sections using the laser capture methods described earlier. Real-time qPCR was carried out using primer sets for Sry (gene of interest) and Cyclophilin, a commonly used housekeeper gene. Sequences used were Rattus norvegicus Sry

(NM 012772.1) and Cyclophilin (M19533.1). Primer sets were designed using Beacon Designer software (Premier Biosoft International, Palo Alto CA.). All sequences were confirmed using the Basic Local Alignment and Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI, Bethesda, MD). Sry primers used were: 5'Sry: 5'- GGG ACA ACA ACC TAC ACA CTA TC -3' and 3'Sry: 5'-CTG GTG CTG CTG TTT CTG C - 3'. Cyclophilin primers used were 5'cyclophilin: 5'- ATC AAA CCA TTC CTT CTG TAG CTC - 3' and 3'cyclophilin: 5' - GGA ACC CAA AGA ACT TCA GTG AG - 3'.

Temperature, primer concentration and DNA concentration were optimized using a Bio-Rad I cycler with a gradient block. Real-time qPCR amplicons were run on a 3% agarose gel to confirm proper size. They were then extracted and sequenced on an Applied Biosystems Incorporated 3730XL DNA analyzer (Foster City, CA) to confirm product. Real-time qPCR reactions were then run using the Bio-Rad MyiQ system with sybr green and melt curve analysis using the following conditions: (i) 3 minutes denaturation at 95 degrees for 1 cycle, (ii) 15 seconds of denaturation at 95 degrees, 1 minute of annealing and extension at 66 degrees for 51 cycles followed by (iii) generation of a melting curve. Melt curves were performed as follows: (i) 1 minute at 95^0C, (ii) 1 minute at 55^0C, (iii) 81 repeats at 55^0C with reading of fluorescence every 10 seconds. Samples that did not contain enough DNA for real-time qPCR after laser capture microdissection were excluded.

Standard Curve

A standard curve was run for both Sry and Cyclophilin using the synthetic amplicon. A standard curve was calculated using linear regression analysis. The amplicon was diluted using 10 fold serial dilutions from 10^1-10^8 molecules/µl. The dynamic range of the curve spanned at least six orders of magnitude. The amount of product in a particular sample was determined by interpolation from a standard curve of Ct values generated from the synthetic amplicon dilution series. Efficiencies were all 90-100%, coefficients 0.990-1.000 and standard curve slopes -3.2 to -3.5.

Data analysis

The raw data from experimental samples produced by the MyiQ real-time instrument and program were transferred to Linereg Software[5] to calculate the efficiency for each well[6]. Relative quantification was determined by calculating the

relative Expression Rate (rER) with the Gene Expression Ct Difference (GED) formula according to Schefe[6]. This calculation takes the individual efficiencies of amplification for each well into account, and allows for normalization to a reference sample (male control). Three threshold cycle values (C_t1, C_t2, and Ct3) were obtained from separate amplification products of each gene, thus producing three rER values for each specimen to verify a normal distribution. On each real-time qPCR run, female and male control samples were also included in triplicate. A total of 8 male and 8 female controls were tested. In each calculation, the male-only control sample served as the reference sample (ref). We averaged these three relative expression ratios (rER), with individual PCR efficiencies (E) included, according to the formula:

$$rER = \frac{R_{norm}(SOI)}{R_{norm}(ref)} = \frac{(1+E(HKG))^{CT(HKG;SOI)-CT(HKG;ref)}}{(1+E(GOI))^{CT(GOI;SOI)-CT(GOI;ref)}}$$

where R_{norm} is the relative quantity of the Gene of interest (GOI: Sry) to the Housekeeper gene (HKG: Cyclophilin) by clustering the corresponding values as indicated in Figure 2. We assumed that the calculated rERs for one sample-of-interest (SOI) are part of a normal distribution (as the C_t and E values are), thus allowing calculation of the mean value and the standard deviation of these rERs[6]. A rER close to 1.0 reflects a predominately recipient (male) derived cell population, while lower rERs represent a majority of surviving donor (female) bone cells. A higher rER thus reflects a higher rate of transplant chimerism.

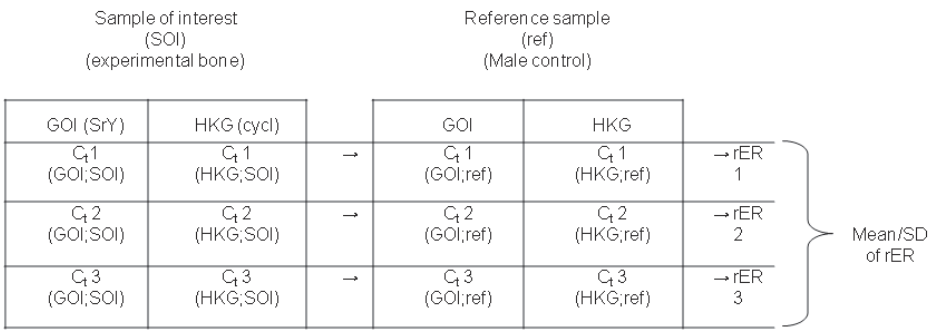

Figure 2. Algorithm for calculation of the relative expression ratio (rER) from the cycle threshold (C_t) values of each sample. Since the measurements were done in triplicates we used 3 values (C_t1, C_t2, C_t3) for each probe.

Statistics

A Kruskal-Wallis test was used to detect differences across the four treatment groups. Pairwise comparisons using the Wilcoxon rank sum test were performed to further clarify the variables driving significant variances. Male and female control data were included.

Results

Bone blood flow was significantly higher in those animals in which both short-term immunosuppression (IS) and a patent AV bundle were used, when compared to groups who received no immunosuppression or had their AV bundles ligated (Figure 3). The median blood flow (range) measured in animals with patent AV bundles with no immunosuppression (Group 1) was 0.00 (0.00 - 0.21) and was 0.12 (0.00 - 0.27) with short-term immunosuppression (Group 3). In those animals with ligated AV bundles, median cortical blood flow measured 0.00 (0.00 - 0.07) (Group 2) and 0.00 (0.00 - 0.09) in Group 4 ($p<0.01$).

Bone viability scores on whole histologic specimens showed that the lowest (best) median value was obtained in the short-term IS and patent AV bundle group (Figure 4).

In determination of transplant chimerism, we excluded samples from which no DNA could be extracted (Table 1). We measured a relative expression ratio (rER) of 1.04 ± 0.06 for male and 0.03 ± 0.04 for female controls (Mean ± SD). These values demonstrate male controls to have essentially equal expression of cyclophilin and Sry genes (100%), while female controls had no Sry expression. The mean rER was lowest in group 4 (0.77 ± 0.06) and highest in group 2 (0.92 ± 0.12), while group 3 had a rER of 0.81 ± 0.05 and group 1 had a rER of 0.88±0.1 (Figure 5).

Only four of seven animals treated with no immunosuppression and a ligated AV bundle (Group 2) had DNA left for analysis, due to bone necrosis (Table 1). When the four groups were compared, a significant difference was found between Groups 1 (88%; no IS; patent AV bundle) and 4 (77%; IS, ligated AV bundle) ($p=0.02$; Figure 5). There was no significant correlation between relative Expression Rates and bone blood flow or histology.

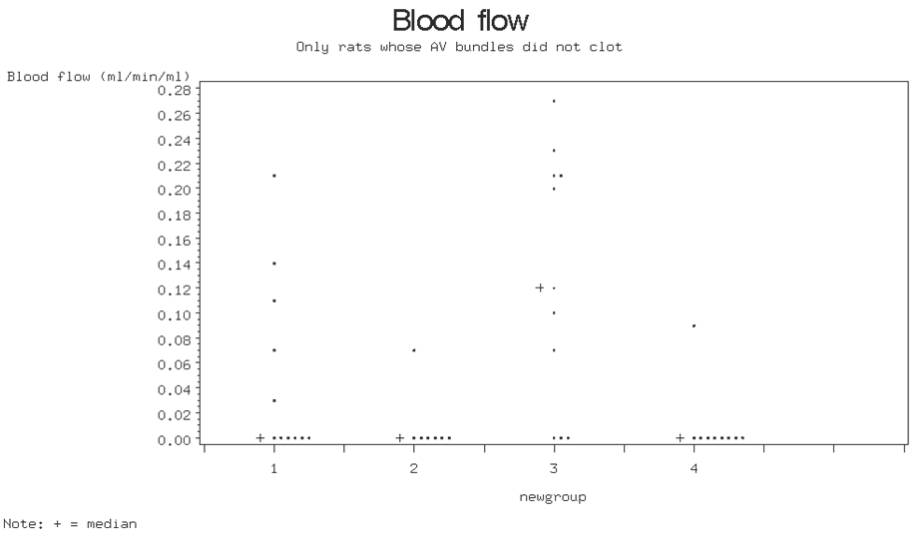

Figure 3. Bone blood flow (mL/min/100 g).

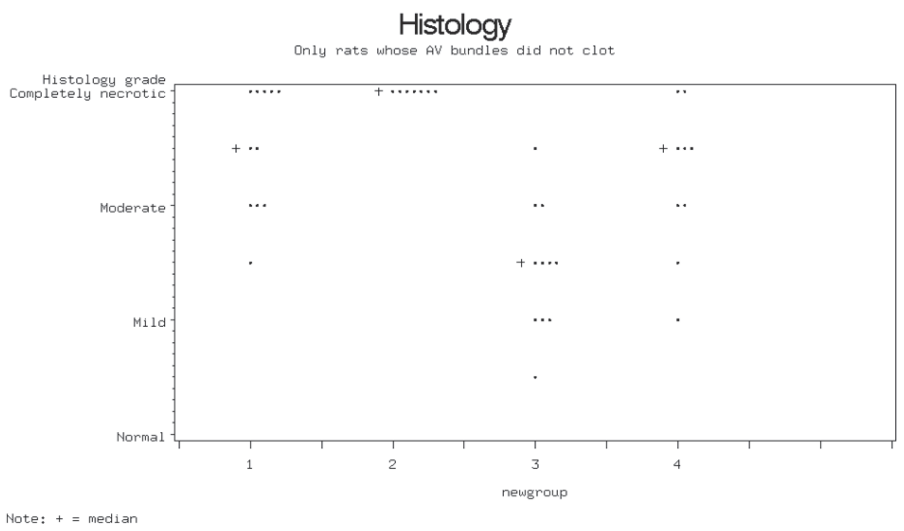

Figure 4. Histological grading of bone viability. Bone necrosis was least severe in Group 3 specimens (with patent AV bundle and Tacrolimus IS).

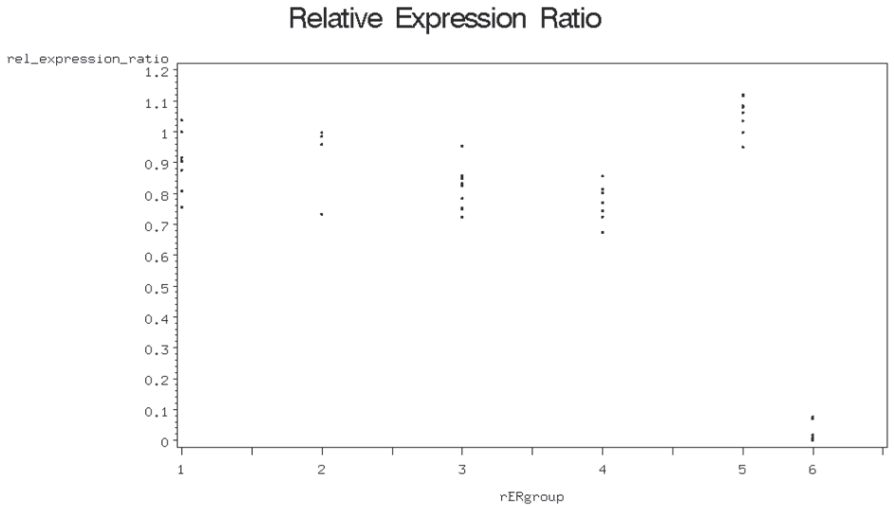

Figure 5. Relative expression ratio in the different groups (5=male; 6= female).

Discussion

Identification of the lineage of cells (recipient or transplant origin) is important in understanding the mechanisms by which viability is maintained in tissue transplantation. In previous work, we employed a competitive polymerase chain reaction, simultaneously amplifying a Y-chromosome-specific gene (Sry) and an autosomal gene to quantify changes in cell populations, after isolating DNA from homogenized whole bone samples. These studies have been conducted in both isotransplants[3,7] and allotransplants[8]. The gender mismatch applied in these models permits the study of cell lineage. Such procedures are common in clinical transplantation, and may contribute to immunogenicity. Examples of poorer outcome exist for both male-to-female and female-to-male transplants[9], although only the former has a proven H-Y antigen minor histocompatibility mismatch.

We have demonstrated that a substantial repopulation of the transplanted rat femora occurs by 18 weeks after transplantation, with cells of recipient lineage (transplant chimerism)[3]. This gradual replacement of donor cells by recipient-derived cells occurs such that by 24 weeks only 10% of the remaining cells are of donor origin as shown in a previous study[7]. These studies confirmed that vascularized bone allotransplants

are largely repopulated over time by cells of recipient origin, but were unable to clarify what type of cells the chimeric male cells were, nor their specific location within the bone[7]. Efforts to determine lineage of single cells with molecular methods in bone can be difficult. For example, in-situ hybridization should allow analysis of individual cells on microscopic inspection. In practice, however, the method is technically challenging in calcified tissue. In this paper, we report a new analysis of bone allotransplant chimerism, using laser capture microdissection to select cells of interest, followed by their analysis using real-time qPCR. Selective sampling of areas of cortical bone should provide better specificity of cell type than use of whole-bone samples.

The identity of viable osteocytes is of particular interest, as this would provide evidence for the underlying mechanism permitting bone survival after the removal of immunosuppression. Should the osteocytes themselves prove to be chimeric, as our data seem to suggest, it would be likely that the transplanted bone was able to successfully maintain its structure and function by first replacing the allogenic endosteal blood supply with a recipient-derived neoangiogenic circulation, followed by a process of remodelling and new bone formation from circulation-derived osteoprogenitor cells. A finding of viable allogeneic osteocytes would imply a different mechanism of transplant survival. For example, in *graft adaptation*[10,11], the transplanted bone may acquire properties that allow it to survive in a non-tolerant but immunologically competent recipient[12]. In osseous tissue, allogeneic matrix and cells may be sequestered by new bone formation, providing protection similar to diminished blood flow[13]. Short-term immunosuppression at times permits longer-term tissue survival after its withdrawal[14,15]. Kuroki et al. demonstrated prolonged limb allotransplant survival in rats after short-term 14 day Tacrolimus therapy[16]. Muramatsu et al. also reported an average 31 day delay in hind limb allotransplant rejection after short-term Tacrolimus therapy (1mg/kg/day for 30 days) in rats[15]. The mechanism that permits tissue survival has been presumed to be sequestration of immunogenic matrix by new bone formation, but this assumption has not been demonstrated experimentally[13,17]. A similar finding would be expected with induction of donor-specific tolerance or with successful drug-mediated immunosuppression.

Some evidence from solid organ transplantation suggests that tolerance is associated with the development of mixed chimerism (survival of transplant lymphocytes in

recipient lymphoid tissue)[18,19], although a causal relationship has not been proven. Future studies of bone and composite tissue allotransplantation should include analysis of recipient animal lymphoid tissue for mixed chimerism, as well as analysis of osteocytes for quantification of transplant chimerism. We believe our methods have considerable potential in answering these important questions. Our results demonstrate that, while varying proportions of remaining donor DNA were identified in all groups, most of the DNA proved to be male (recipient derived) in origin. Our samples, obtained by laser capture microdissection typically included a relatively large number of cells. While efforts to sample osteocytes alone was the goal, it is possible that the samples included other types of recipient-derived (male) cells, DNA remnants from both male and female nonviable cells as well as surviving transplanted female cells. We believe that the lineage results demonstrate an ongoing remodelling process, however, mediated primarily by recipient animal cells via the recipient-derived neoangiogenic blood supply.

Short-term Tacrolimus immunosuppression seems to play a key role in maintaining osteocyte viability during development of a neoangiogenic circulation using our bone transplantation method. This is reflected in the histologic scores. For example, Group 4 (IS; ligated AV bundle) transplants have no blood supply after withdrawal of the immunosuppression, and so have fewer viable cells than Group 3. Furthermore, while only Group 4 had a significantly lower rER than Group 1, Groups 3 and 4 both reveal a trend towards a lower rER than Groups 1 and 2, presumably due to the use of immunosuppression. The difference between Groups 4 and 2 emphasizes this trend. The lack of statistical significance is due at least in part to the reduced sample size in Group 2.

These results indicate that short term immunosuppression may cause relatively less donor cell death resulting in decreased repopulation of the transplant by male recipient cells. However, this protection of donor cell viability by immunosuppression seems to be undermined by the presence of a patent recipient arteriovenous bundle, reflected by the higher rER in Group 3 compared to Group 4. Long term maintenance of blood supply may result in increased exposure of donor cell surface alloantigens to the recipient immune system, resulting in increased rejection of donor cells. This could allow for more repopulation with recipient cells whose migration is facilitated by the neoangiogenic recipient blood supply.

Further study will be required, with identification and sampling of osteocytes in regions of new cortical bone formed following transplantation. It may be that bone formation follows a bi-modal distribution, beginning initially by surviving allogeneic osteoblasts, but eventually predominated by osteogenesis from recipient-derived cells invading the transplant[20]. With our unique transplantation method, one might expect the microsurgical anastomoses in animals receiving no immunosuppression to thrombose quickly, and the female osteocytes to die. Thus, most DNA isolated from the bone would be from viable male cells engaged in creeping substitution of necrotic bone. Our histologic and lineage data would support this hypothesis. All other groups would likely have at least some female (transplanted) cells surviving, via nutrition provided from either a patent AV bundle (if not ligated), a patent nutrient vessel (if IS used), or both. This is indeed what our data suggest. A more detailed study performed with multiple fluorochrome labeling at early and late time points would help to demonstrate if such a bimodal distribution of osteocyte lineage can be found in femora treated with both IS and AV-bundle implantation.

Acknowledgement

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